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Title of Invention	Antisense Oligonucleotides Directed to Ribonucleotide Reductase R1 and Uses Thereof in the Treatment of Cancer

Application Number:
Date:
First Named Applicant: Aiping H. Young

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	us-request.dtd
	us-request.xsl
us-fee-sheet	MBM-usfees.xml
	us-fee-sheet.xsl
	us-fee-sheet.dtd
application-body	spev-trans.xml

us-application-body.xsl
application-body.dtd
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mathml2-qname-1.mod
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Electronic Version

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Description

Antisense oligonucleotides directed to ribonucleotide reductase R1 and uses thereof in the treatment of cancer

BACKGROUND OF INVENTION

[0001] The present invention pertains to the field of cancer therapeutics and in particular to compositions of an antisense oligonucleotide alone or in combination with one or more chemotherapeutic drugs for the treatment of cancer.

[0002] The first unique step leading to DNA synthesis is the conversion of ribonucleotides to their corresponding deoxyribonucleotides, a reaction that is catalyzed in a cell cycle specific manner by the housekeeping gene ribonucleotide reductase [Lewis et al., 1978; Reichard, 1993; Wright, 1989a; Wright et al., 1990a; Stubbe, 1989]. The mammalian enzyme is composed of two dissimilar dimeric protein components often called R1 and R2, which are encoded by two different genes located on different chromosomes [Bjorklund et al., 1993; Tonin et al., 1987].

[0003] The levels of the R1 protein do not appear to change substantially during the cell cycle of proliferating cells and can be detected.

throughout the cell cycle. Synthesis of R1 mRNA, like R2 mRNA appears to occur mainly during S phase [Eriksson et al., 1984; Choy et al., 1988; Mann et al., 1988]. The broader distribution of the R1 protein during the cell cycle is attributed to its longer half life as compared to the R2 protein [Choy et al., 1988; Mann et al., 1988].

[0004] Regulation of ribonucleotide reductase, and particularly the R2 component, is altered in malignant cells exposed to some tumour promoters and to the growth factor TGF-Î² [Amara, et al., 1994; Chen et al., 1993; Amara et al., 1995b; Hurta and Wright, 1995; Hurta et al., 1991]. Higher levels of enzyme activity have been observed in cultured malignant cells when compared to nonmalignant cells [Weber, 1983; Takeda and Weber, 1981; Wright et al., 1989a], and increased levels of R2 protein and R2 mRNA have been found in pre-malignant and malignant tissues as compared to normal control tissue samples [Saeki et al., 1995; Jensen et al., 1994].

[0005] However, these correlative studies did not show a direct role for ribonucleotide reductase in cancer cell transformation and tumor progression, because like so many other enzyme activities found to be altered in cancer cells [e.g. Weber, 1983], the results could easily be explained by the increased cell proliferation and altered cell cycle regulation characteristics of transformed and malignant cell populations [Morgan and Kastan, 1997].

[0006] Antisense oligonucleotides directed to the R1 or R2 component of ribonucleotide reductase have been shown to be effective in reducing the growth of cancer cells [see, for example, U.S. Patent Nos. 5,998,383 and 6,121,000].

[0007] In view of the high incidence of various types of cancer throughout the world; there remains a need for improved therapies for the treatment of cancer.

[0008] This background information is provided for the purpose of making known information believed by the applicant to be of possible relevance to the present invention. No admission is necessarily intended, nor should be construed, that any of the preceding information constitutes prior art against the present invention. Publications referred to throughout the specification are hereby incorporated by reference in their entireties in this application.

SUMMARY OF INVENTION

[0009] An object of the present invention is to provide antisense oligonucleotides directed to ribonucleotide reductase R1 and uses thereof in the treatment of cancer. In accordance with an aspect of the present invention, there is provided a use of an antisense oligonucleotide comprising SEQ ID NO:1 alone or in combination with a chemotherapeutic for the treatment of prostate cancer.

BRIEF DESCRIPTION OF DRAWINGS

[0010]

Figure 1 depicts the effects of the nucleotide sequence according to

SEQ ID NO:1 on PC-3 and DU145 Prostate Tumor Growth in SCID Mice. Treatment with SEQ ID NO:1 demonstrated a strong inhibitory effect on the growth of human prostate carcinoma.

[0011] Figure 2 depicts the effects of the nucleotide sequence according to SEQ ID NO:1 on DU145 Prostate Tumor Growth in SCID Mice. The anti-tumor effect of SEQ ID NO:1 was further compared to that of mitoxantrone (novantrone®) alone or in combination.

[0012] Figure 3 depicts the effects of anti-tumor activity of SEQ ID NO:1 on Caki-1 Human Kidney Tumor Growth in in SCID/beige mice that are NK, T and B cell deficient.

[0013] Figure 4 depicts the effects of SEQ ID NO:1 on R1 mRNA levels in HT-29 colon tumors in CD1 nude mice having HT-29 xenografts.

[0014] Figure 5 depicts measurements of R1 protein levels using Western blot analysis and AD 203, an anti-R1-antibody, in untreated cancer cell lines derived from diverse human cancer types, including renal (Caki 1) and A498, skin (A2058), colon (HT-29) and breast (MDS-MB-231) cancer cell lines. The R1 protein expression was compared to R1 expression in 2 normal cell lines, WI38 and HUVEC.

[0015] Figure 6 depicts the effect of SEQ ID NO:1 on the colony forming ability in the human tumor cell lines, Hep G2 (liver), SK-OV-3 (ovary), U-87 MG (brain), A2058 (melanoma), H460 (lung), MDA-MB-231 (breast) and AsPC-1 (pancreas).

[0016] Figure 7 depicts a Northern blot analysis of the effect of SEQ ID NO:1 on R1 mRNA levels in the human tumor cell lines HT-29 (human colon adenocarcinoma) and MDA-MB-231 (human breast adenocarcinoma) cell lines.

[0017] Figure 8 depicts the effect of SEQ ID NO:1 on the inhibition of the R1 target at the protein level in AsPC-1 human tumor cells (pancreatic adenocarcinoma) using immunoprecipitation analyses.

[0018] Figure 9 depicts the effect of SEQ ID NO:1 on the inhibition of the R1 target at the protein level in MDA-MB-231 human breast adenocarcinoma using immunoprecipitation analyses.

[0019] Figure 10 depicts a Northern blot analyses of other cellular RNA levels in A2058 human melanoma cells treated with SEQ ID NO:1 or a scrambled control analogue of SEQ ID NO:1 in order to examine the specificity of inhibition of R1 mRNA by SEQ ID NO:1.

DETAILED DESCRIPTION

[0020]

The present invention relates to compositions of antisense oligonucleotides directed to a gene encoding a mammalian ribonucleotide reductase R1 protein and one or more chemotherapeutic agents. The compositions of the present invention are useful in the treatment of cancer. The compositions of the antisense oligonucleotides alone or in combination with one or more chemotherapeutic agents has been found to be more effective in decreasing the growth and/or metastasis of cancer

cells, including drug resistant cancer cells, than treatment with the antisense oligonucleotide or the chemotherapeutic agent(s) alone.

[0021] *Definitions*

[0022] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains.

[0023] The term "antisense oligonucleotide"as used herein means a nucleotide sequence that is complementary to the mRNA for the desired gene. In the context of the present invention, the desired gene is the gene encoding a mammalian ribonucleotide redustase R1 protein.

[0024] The term "selectively hybridise"as used herein refers to the ability of a nucleic acid to bind detectably and specifically to a second nucleic acid. Oligonucleotides selectively hybridise to target nucleic acid strands under hybridisation and wash conditions that minimise appreciable amounts of detectable binding to non-specific nucleic acids. High stringency conditions can be used to achieve selective hybridisation conditions as known in the art and discussed herein.

[0025] Typically, hybridisation and washing conditions are performed at high stringency according to conventional hybridisation procedures. Washing conditions are typically 1-3 x SSC, 0.1-1% SDS, 50-70^{O}C with a change of wash solution after about 5-30 minutes.

[0026] The term "corresponds to" as used herein with reference to nucleic

acid sequences means a polynucleotide sequence that is identical to all or a portion of a reference polynucleotide sequence. In contradistinction, the term "complementary to" is used herein to mean that the polynucleotide sequence is identical to all or a portion of the complement of a reference polynucleotide sequence. For illustration, the nucleotide sequence "TATAC"corresponds to a reference sequence "TATAC" and is complementary to a reference sequence "GTATA".

[0027]

The following terms are used herein to describe the sequence relationships between two or more polynucleotides: "reference sequence,""comparison window,""sequence identity,""percentage of sequence identity,"and "substantial identity."A "reference sequence"is a defined sequence used as a basis for a sequence comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length cDNA or gene sequence, or may comprise a complete cDNA or gene sequence. Generally, a reference sequence is at least 20 nucleotides in length, frequently at least 25 nucleotides in length, and often at least 50 nucleotides in length. Since two polynucleotides may each (1) comprise a sequence (*i.e.* a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) may further comprise a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by

comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity.

[0028] A "comparison window", as used herein, refers to a conceptual segment of at least 20 contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a reference sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e. gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith and Waterman (1981) Adv. Appl. Math. *2*:482, by the homology alignment algorithm of Needleman and Wunsch (1970) J. Mol. Biol. *48*:443, by the search for similarity method of Pearson and Lipman (1988) Proc. Natl. Acad. Sci. (U.S.A.) *85*:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 573 Science Dr., Madison, WI), or by inspection, and the best alignment (i.e. resulting in the highest percentage of identity over the comparison window) generated by the various methods is selected.

[0029] The term "sequence identity" means that two polynucleotide

sequences are identical (i.e. on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g. A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e. the window size), and multiplying the result by 100 to yield the percentage of sequence identity.

[0030] The term "substantial identity" as used herein denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 30 percent sequence identity, often at least 50 percent sequence identity, and more usually at least 60 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, and frequently over a window of at least 25-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison.

[0031] *ANTISENSE MOLECULES*

[0032] *Selection and Characteristics*

[0033] It is preferred to target specific nucleic acids for antisense. "Targeting"an antisense compound to a particular nucleic acid, in the context of the present invention, is a multistep process. The process usually begins with the identification of a nucleic acid sequence whose function is to be modulated. This may be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, the target is the gene encoding a mammalian ribonucleotide reductase R1 protein. The sequences of various mammalian ribonucleotide reductase genes are known in the art, for example, the sequence for the human ribonucleotide reductase R1 gene is provided in Bjorklund et al. [P.N.A.S. USA, 90:11322-11326 (1993)]. This and other mammalian R1 sequences are also available from the GenBank database maintained by the NCBI.

[0034] The targeting process also includes determination of a site or sites within this gene for the antisense interaction to occur such that the desired effect, e.g. detection or modulation of expression of the protein encoded by the gene, will result. Once the target site or sites have been identified, oligonucleotides are chosen that are sufficiently complementary (i.e. hybridise with sufficient strength and specificity) to the target to give the desired result.

[0035] Generally, antisense oligonucleotides are targeted to the 5â

untranslated region (5â□-UTR), the translation initiation or start codon region, the open reading frame (ORF), the translation termination or stop codon region or the 3â□ untranslated region (3â□-UTR) of a gene. In accordance with the present invention, the antisense oligonucleotide is targeted to part of the open reading frame region of the gene.

[0036] The antisense oligonucleotides in accordance with the present invention are selected from a sequence complementary to the ribonucleotide reductase R1 gene such that the sequence exhibits the least likelihood of forming duplexes, hair-pins, or of containing homooligomer / sequence repeats. The oligonucleotide may further contain a GC clamp. One skilled in the art will appreciate that these properties can be determined qualitatively using various computer modelling programs, for example, the program OLIGO$^{Â®}$ Primer Analysis Software, Version 5.0 (distributed by National Biosciences, Inc., Plymouth, MN).

[0037] It is understood in the art that an antisense oligonucleotide need not have 100% identity with the complement of its target sequence. The antisense oligonucleotides in accordance with the present invention have a sequence that is at least about 75% identical to the complement of target sequence. In one embodiment of the present invention, the antisense oligonucleotides have a sequence that is at least about 90% identical to the complement of the target sequence. In a related embodiment, they have a sequence that is

at least about 95% identical to the complement of target sequence, allowing for gaps or mismatches of several bases. Identity can be determined, for example, by using the BLASTN program of the University of Wisconsin Computer Group (GCG) software or provided on the NCBI website.

[0038] In order to be effective, antisense oligonucleotides are typically between 7 and 100 nucleotides in length. In one embodiment of the present invention, the antisense oligonucleotides are between about 7 to about 50 nucleotides in length. In other embodiments, the antisense oligonucleotides are between about 7 to about 35 nucleotides in length, between about 15 to about 25 nucleotides in length, and about 20 nucleotides in length.

[0039] The antisense oligonucleotides of the present invention comprise at least 7 contiguous nucleotides, or nucleotide analogues that correspond to a part of the coding region of a mammalian ribonucleotide reductase R1 gene.

[0040] Suitable antisense oligonucleotides for use according to the present invention include those disclosed in U.S. Patent Nos. 5,998,383 and 6,121,000 (herein incorporated by reference) which are targeted to the ribonucleotide reductase R1 gene. In one embodiment of the present invention, the antisense oligonucleotide comprises at least 7 consecutive nucleotides, or nucleotide analogues selected from the antisense oligonucleotide sequence AS-I-618-20:

[0041] 5â - CTC TAG CGT CTT AAA GCC GA -3â [SEQ ID NO:1]

[0042] The term "antisense oligonucleotides" as used herein includes other oligomeric antisense compounds, including oligonucleotide mimetics, modified oligonucleotides, and chimeric antisense compounds. Chimeric antisense compounds are antisense compounds that contain two or more chemically distinct regions, each made up of at least one monomer unit.

[0043] Thus, in the context of this invention, the term "oligonucleotide"refers to an oligomer or polymer of ribonucleic acid (RNA), deoxyribonucleic acid (DNA), or RNA or DNA mimetics. This term, therefore, includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions, which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

[0044] As is known in the art, a nucleoside is a base-sugar combination and a nucleotide is a nucleoside that further includes a phosphate group covalently linked to the sugar portion of the nucleoside. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound, with the normal linkage or backbone of RNA and DNA

being a 3' to 5' phosphodiester linkage. Specific examples of antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include both those that retain a phosphorus atom in the backbone and those that lack a phosphorus atom in the backbone. For the purposes of the present invention, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

[0045]

Exemplary modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included. In one embodiment of the present invention, the antisense

oligonucleotide comprises at least one phosphorothioate linkage.

[0046] Exemplary modified oligonucleotide backbones that do not include a phosphorus atom are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. Such backbones include morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulphone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulphamate backbones; methyleneimino and methylenehydrazino backbones; sulphonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

[0047] The present invention also contemplates oligonucleotide mimetics in which both the sugar and the internucleoside linkage of the nucleotide units are replaced with novel groups. The base units are maintained for hybridisation with an appropriate nucleic acid target compound. An example of such an oligonucleotide mimetic, which has been shown to have excellent hybridisation properties, is a peptide nucleic acid (PNA) [Nielsen et al., Science, *254*:1497-1500 (1991)]. In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are

retained and are bound directly or indirectly to aza-nitrogen atoms of the amide portion of the backbone.

[0048]

Modified oligonucleotides may also contain one or more substituted sugar moieties. For example, oligonucleotides may comprise sugars with one of the following substituents at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Examples of such groups are: $O[(CH_2)_n O]_m CH_3$, $O(CH_2)_n OCH_3$, $O(CH_2)_n NH_2$, $O(CH_2)_n CH_3$, $O(CH_2)_n ONH_2$, and $O(CH_2)_n ON[(CH_2)_n CH_3)]_2$, where n and m are from 1 to about 10. Alternatively, the oligonucleotides may comprise one of the following substituents at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, $SO_2 CH_3$, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. Specific examples include 2'-methoxyethoxy (2'-O--$CH_2 CH_2 OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) [Martin et al., Helv. Chim. Acta, *78*:486-504(1995)], 2'-dimethylaminooxyethoxy ($O(CH_2)_2 ON(CH_3)$

$_2$ group, also known as 2'-DMAOE), 2'-methoxy (2'-O--CH_3), 2'-aminopropoxy (2'-OCH_2 CH_2 CH_2 NH_2) and 2'-fluoro (2'-F). In one embodiment of the present invention, the antisense oligonucleotide comprises at least one nucleotide comprising a substituted sugar moiety. In another embodiment, the antisense oligonucleotide comprises at least one 2'-O-(2-methoxyethyl) or 2'-MOE modified nucleotide.

[0049] Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar.

[0050] Oligonucleotides may also include modifications or substitutions to the nucleobase. As used herein, "unmodified"or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5- hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil

(pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in U.S. Pat. No. 3,687,808; The Concise Encyclopedia Of Polymer Science And Engineering, (1990) pp 858-859, Kroschwitz, J. I., ed. John Wiley & Sons; Englisch et al., Angewandte Chemie, Int. Ed., *30*:613 (1991); and Sanghvi, Y. S., (1993) Antisense Research and Applications, pp 289-302, Crooke, S. T. and Lebleu, B., ed., CRC Press. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2Â°C [Sanghvi, Y. S., (1993) Antisense Research and Applications, pp 276-278, Crooke, S. T. and Lebleu, B., ed., CRC Press, Boca Raton].

[0051]

Another oligonucleotide modification included in the present invention is the chemically linkage to the oligonucleotide of one or more moieties or conjugates which enhance the activity, cellular

distribution or cellular uptake of the oligonucleotide. Such moieties include, but are not limited to, lipid moieties such as a cholesterol moiety [Letsinger et al., Proc. Natl. Acad. Sci. USA, *86*:6553-6556 (1989)], cholic acid [Manoharan et al., Bioorg. Med. Chem. Let., *4*:1053-1060 (1994)], a thioether, e.g. hexyl-S-tritylthiol [Manoharan et al., Ann. N.Y. Acad. Sci., *660*:306-309 (1992); Manoharan et al., Bioorg. Med. Chem. Lett., *3*:2765-2770 (1993)], a thiocholesterol [Oberhauser et al., Nucl. Acids Res., *20*:533-538 (1992)], an aliphatic chain, *e.g.* dodecandiol or undecyl residues [Saison-Behmoaras et al., EMBO J., *10*:1111-1118 (1991); Kabanov et al., FEBS Lett., *259*:327-330 (1990); Svinarchuk et al., Biochimie, *75*:49-54 (1993)], a phospholipid, *e.g.* di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate [Manoharan et al., Tetrahedron Lett., *36*:3651-3654 (1995); Shea et al., Nucl. Acids Res., *18*:3777-3783 (1990)], a polyamine or a polyethylene glycol chain [Manoharan et al., Nucleosides & Nucleotides, *14*:969-973 (1995)], or adamantane acetic acid [Manoharan et al., Tetrahedron Lett., *36*:3651-3654 (1995)], a palmityl moiety [Mishra et al., Biochim. Biophys. Acta, *1264*:229-237 (1995)], or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety [Crooke et al., J. Pharmacol. Exp. Ther., *277*:923-937 (1996)].

[0052]

One skilled in the art will recognise that it is not necessary for all positions in a given oligonucleotide to be uniformly modified. The

present invention, therefore, contemplates the incorporation of more than one of the aforementioned modifications into a single oligonucleotide or even at a single nucleoside within the oligonucleotide. The present invention further includes antisense compounds that are chimeric compounds. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease that cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridising to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridisation techniques known in the art.

[0053]

An example of a suitable chimeric oligonucleotide would be an antisense oligonucleotides with a mixed phosphorothioate and

2â□3-O-methyl backbone. In one embodiment of the present invention, the antisense oligonucleotides comprise one or more phosphorothioate backbone linkages. In a another embodiment, all backbone linkages in the antisense oligonucleotide are phosphorothioate linkages.

[0054] In the context of the present invention, an antisense oligonucleotide is "nuclease resistant" when it has either been modified such that it is not susceptible to degradation by DNA and RNA nucleases or alternatively has been placed in a delivery vehicle which in itself protects the oligonucleotide from DNA or RNA nucleases. Nuclease resistant oligonucleotides include, for example, methyl phosphonates, phosphorothioates, phosphorodithioates, phosphotriesters, and morpholino oligomers. Suitable delivery vehicles for conferring nuclease resistance include, for example, liposomes. In one embodiment of the present invention, the antisense oligonucleotides are nuclease resistant.

[0055] The present invention further contemplates antisense oligonucleotides that contain groups for improving the pharmacokinetic properties of the oligonucleotide, or groups for improving the pharmacodynamic properties of the oligonucleotide.

[0056] *Preparation of the Antisense Oligonucleotides*

[0057]

The antisense oligonucleotides of the present invention can be prepared by conventional techniques well-known to those skilled in

the art. For example, the oligonucleotides can be prepared using solid-phase synthesis using commercially available equipment, such as the equipment available from Applied Biosystems Canada Inc., Mississauga, Canada. As is well-known in the art, modified oligonucleotides, such as phosphorothioates and alkylated derivatives, can also be readily prepared by similar methods.

[0058] Alternatively, the antisense oligonucleotides of the present invention can be prepared by enzymatic digestion of the naturally occurring ribonucelotide reductase R1 gene by methods known in the art.

[0059] Antisense oligonucleotides can also be prepared through the use of recombinant methods in which expression vectors comprising nucleic acid sequences that encode the antisense oligonucleotides are expressed in a suitable host cell. Such expression vectors can be readily constructed using procedures known in the art. Examples of suitable vectors include, but are not limited to, plasmids, phagemids, cosmids, bacteriophages, baculoviruses and retroviruses, and DNA viruses. One skilled in the art will understand that selection of the appropriate host cell for expression of the antisense oligonucleotide will be dependent upon the vector chosen. Examples of host cells include, but are not limited to, bacterial, yeast, insect, plant and mammalian cells.

[0060] One skilled in the art will also understand that the expression vector may further include regulatory elements, such as transcriptional

elements, required for efficient transcription of the antisense oligonucleotide sequences. Examples of regulatory elements that can be incorporated into the vector include, but are not limited to, promoters, enhancers, terminators, and polyadenylation signals. One skilled in the art will appreciate that selection of suitable regulatory elements is dependent on the host cell chosen for expression of the antisense oligonucleotide and that such regulatory elements may be derived from a variety of sources, including bacterial, fungal, viral, mammalian or insect genes.

[0061] In accordance with the present invention, the expression vectors can be introduced into a suitable host cell or tissue by one of a variety of methods known in the art. Such methods can be found generally described in Sambrook et al., 1992; Ausubel et al., 1989; Chang et al., 1995; Vega et al., 1995; and Vectors: A Survey of Molecular Cloning Vectors and Their Uses (1988) and include, for example, stable or transient transfection, lipofection, electroporation, and infection with recombinant viral vectors.

[0062] *CHEMOTHERAPEUTIC AGENTS*

[0063] The combinations provided by the present invention comprise an antisense oligonucleotide and one or more chemotherapeutic agents. A wide range of cancer chemotherapeutic agents is known in the art and includes those chemotherapeutic agents which are specific for the treatment of a particular type of cancer as well as those which may be applicable to a range of cancers, such as

doxorubicin, capecitabine, mitoxantrone, irinotecan (CPT-11). The present invention contemplates the use of both types of chemotherapeutic agent in conjunction with the antisense oligonucleotides. Combination therapies using standard cancer chemotherapeutics are also well known in the art and may be used in conjunction with the antisense oligonucleotides.

[0064] Examples of chemotherapeutic agents suitable for the treatment of breast cancer include, but are not limited to, capecitabine, cyclophosphamide, ifosfamide, cisplatin, carboplatin, 5-fluorouracil (5-FU), taxol, taxanes such as paclitaxel and docetaxel and various anthracyclines, such as doxorubicin and epi-doxorubicin (also known as epirubicin). Combination therapies using standard cancer chemotherapeutics may also be used in conjunction with the antisense oligonucleotides and are also well known in the art, for example, the combination of epirubicin with paclitaxel or docetaxel, or the combination of doxorubicin or epirubicin with cyclophosphamide, which are used for breast cancer treatments. Polychemotherapeutic regimens are also useful and may consist, for example, of doxorubicin/cyclophosphamide/5-fluorouracil or cyclophosphamide/epirubicin/5-fluorouracil. Many of the above chemotherapeutics and combinations thereof are useful in the treatment of a variety of solid tumours.

[0065] Cyclophosphamide, mitoxantrone and estramustine are known to be suitable for the treatment of prostate cancer.

Cyclophosphamide, vincristine, doxorubicin and etoposide are used in the treatment of small cell lung cancer, as are combinations of etoposide with either cisplatin or carboplatin. In the treatment of stomach or oesophageal cancer, combinations of doxorubicin or epirubicin with cisplatin and 5-fluorouracil are useful. For colorectal cancer, CPT-11 alone or in combination with 5-fluorouracil-based drugs, or oxaliplatin alone or in combination with 5-fluorouracil-based drugs can be used. Oxaliplatin may also be usedin combination with capecitabine.

[0066] Other examples include the combination of cyclophosphamide, doxorubicin, vincristine and prednisone in the treatment of non-Hodgkin"s lymphoma; the combination of doxorubicin, bleomycin, vinblastine and DTIC in the treatment of Hodgkin"s disease and the combination of cisplatin or carboplatin with any one or a combination of gemcitabine, paclitaxel, docetaxel, vinorelbine or etoposide in the treatment of non-small cell lung cancer.

[0067] Other suitable chemotherapeutic agents include, but are not limited to, mitomycin C, vinblastine, 3,4 anhydrovinblastine, Navelbine, IL-2-II and IL-2-I, novantrone, DTIC, hydroxyurea, PALA and methotrexate (MTX).

[0068] Examples of suitable combinations of the antisense oligonucleotide and one or more chemotherapeutic agent include, but are not limited to, a combination of the antisense oligonucleotide and capecitabine for the treatment of solid tumours, breast cancer, renal

cancer or colorectal cancer; a combination of the antisense oligonucleotide, capecitabine and oxaliplatin for the treatment of colorectal cancer and pancreatic cancer; a combination of the antisense oligonucleotide and docetaxel for the treatment of solid tumours, including non-small cell lung carcinoma (NSCLC), prostate cancer and cancer of the genitourinary tract; a combination of the antisense oligonucleotide and gemcitabine for the treatment of solid tumours, NSCLC and renal cell carcinoma; a combination of the antisense oligonucleotide, gemcitabine and capecitabine for the treatment of colon cancer; a combination of the antisense oligonucleotide, gemcitabine and oxaliplatin for the treatment of breast cancer; a combination of the antisense oligonucleotide and idarubicin for the treatment of acute myeloid leukaemia (AML); a combination of the antisense oligonucleotide and Ara-C for the treatment of AML and chronic myeloid leukaemia (CML); a combination of the antisense oligonucleotide, mitoxantrone, etoposide and Ara-C for the treatment of AML; a combination of the antisense oligonucleotide, fludarabin, filgrastim and Ara-C for the treatment of CML; a combination of the antisense oligonucleotide, carboplatin and paclitaxel for the treatment of metastatic cancer; a combination of the antisense oligonucleotide and cisplatin for the treatment of head and neck cancer, oesophageal cancer and lung cancer; a combination of the antisense oligonucleotide, cisplatin and irinotecan for the treatment of small-cell lung carcinoma (SCLC); a combination of the antisense oligonucleotide and

irinotecan for the treatment of pancreatic adenocarcinoma, and a combination of the antisense oligonucleotide and 5-FU for the treatment of cancer of the pancreas, gall bladder and biliary ducts.

[0069] *USE OF THE COMPOSITIONS OF THE PRESENT INVENTION*

[0070] The compositions of the present invention comprising an antisense oligonucleotide directed to a ribonucleotide reductase R1 gene alone or in combination with one or more chemotherapeutic agents can be used in the treatment of a variety of cancers. In accordance with the present invention, the compositions are effective in reducing the growth and/or metastasis of cancer cells and can also be used to effectively treat drug resistant tumours.

[0071] Examples of cancers which may be may be treated, stabilised, or prevented in accordance with the present invention include, but are not limited to leukaemia, carcinomas, adenocarcinomas, melanomas and sarcomas. Carcinomas, adenocarcinomas and sarcomas are also frequently referred to as "solid tumors," examples of commonly occurring solid tumors include, but are not limited to, cancer of the brain, breast, cervix, colon, head and neck, kidney, lung, ovary, pancreas, prostate, stomach and uterus, non-small cell lung cancer and colorectal cancer.

[0072] The term "leukaemia" refers broadly to progressive, malignant diseases of the blood-forming organs. Leukaemia is typically characterised by a distorted proliferation and development of

leukocytes and their precursors in the blood and bone marrow but can also refer to malignant diseases of other blood cells such as erythroleukaemia, which affects immature red blood cells. Leukaemia is generally clinically classified on the basis of (1) the duration and character of the disease acute or chronic; (2) the type of cell involved myeloid (myelogenous), lymphoid (lymphogenous) or monocytic, and (3) the increase or non-increase in the number of abnormal cells in the blood leukaemic or aleukaemic (subleukaemic). Leukaemia includes, for example, acute nonlymphocytic leukaemia, chronic lymphocytic leukaemia, acute granulocytic leukaemia, chronic granulocytic leukaemia, acute promyelocytic leukaemia, adult T-cell leukaemia, aleukaemic leukaemia, aleukocythemic leukaemia, basophylic leukaemia, blast cell leukaemia, bovine leukaemia, chronic myelocytic leukaemia, leukaemia cutis, embryonal leukaemia, eosinophilic leukaemia, Gross" leukaemia, hairy-cell leukaemia, hemoblastic leukaemia, hemocytoblastic leukaemia, histiocytic leukaemia, stem cell leukaemia, acute monocytic leukaemia, leukopenic leukaemia, lymphatic leukaemia, lymphoblastic leukaemia, lymphocytic leukaemia, lymphogenous leukaemia, lymphoid leukaemia, lymphosarcoma cell leukaemia, mast cell leukaemia, megakaryocytic leukaemia, micromyeloblastic leukaemia, monocytic leukaemia, myeloblastic leukaemia, myelocytic leukaemia, myeloid granulocytic leukaemia, myelomonocytic leukaemia, Naegeli leukaemia, plasma cell leukaemia, plasmacytic

leukaemia, promyelocytic leukaemia, Rieder cell leukaemia, Schilling's leukaemia, stem cell leukaemia, subleukaemic leukaemia, and undifferentiated cell leukaemia.

[0073] The term "sarcoma"generally refers to a tumor which originates in connective tissue, such as muscle, bone, cartilage or fat, and is made up of a substance like embryonic connective tissue and is generally composed of closely packed cells embedded in a fibrillar or homogeneous substance. Sarcomas include soft tissue sarcomas, chondrosarcoma, fibrosarcoma, lymphosarcoma, melanosarcoma, myxosarcoma, osteosarcoma, Abemethy"s sarcoma, adipose sarcoma, liposarcoma, alveolar soft part sarcoma, ameloblastic sarcoma, botryoid sarcoma, chloroma sarcoma, chorio carcinoma, embryonal sarcoma, Wilms" tumor sarcoma, endometrial sarcoma, stromal sarcoma, Ewing"s sarcoma, fascial sarcoma, fibroblastic sarcoma, giant cell sarcoma, granulocytic sarcoma, Hodgkin"s sarcoma, idiopathic multiple pigmented haemorrhagic sarcoma, immunoblastic sarcoma of B cells, lymphoma, immunoblastic sarcoma of T-cells, Jensen"s sarcoma, Kaposi"s sarcoma, Kupffer cell sarcoma, angiosarcoma, leukosarcoma, malignant mesenchymoma sarcoma, parosteal sarcoma, reticulocytic sarcoma, Rous sarcoma, serocystic sarcoma, synovial sarcoma, and telangiectaltic sarcoma.

[0074] The term "melanoma"is taken to mean a tumor arising from the melanocytic system of the skin and other organs. Melanomas

include, for example, acral-lentiginous melanoma, amelanotic melanoma, benign juvenile melanoma, Cloudman"s melanoma, S91 melanoma, Harding-Passey melanoma, juvenile melanoma, lentigo maligna melanoma, malignant melanoma, nodular melanoma, subungal melanoma, and superficial spreading melanoma.

[0075]

The term "carcinoma"refers to a malignant new growth made up of epithelial cells tending to infiltrate the surrounding tissues and give rise to metastases. Exemplary carcinomas include, for example, acinar carcinoma, acinous carcinoma, adenocystic carcinoma, adenoid cystic carcinoma, carcinoma adenomatosum, carcinoma of adrenal cortex, alveolar carcinoma, alveolar cell carcinoma, basal cell carcinoma, carcinoma basocellulare, basaloid carcinoma, basosquamous cell carcinoma, bronchioalveolar carcinoma, bronchiolar carcinoma, bronchogenic carcinoma, cerebriform carcinoma, cholangiocellular carcinoma, chorionic carcinoma, colorectal carcinoma, colloid carcinoma, comedo carcinoma, corpus carcinoma, cribriform carcinoma, carcinoma en cuirasse, carcinoma cutaneum, cylindrical carcinoma, cylindrical cell carcinoma, duct carcinoma, carcinoma durum, embryonal carcinoma, encephaloid carcinoma, epiermoid carcinoma, carcinoma epitheliale adenoides, exophytic carcinoma, carcinoma ex ulcere, carcinoma fibrosum, gelatiniform carcinoma, gelatinous carcinoma, giant cell carcinoma, carcinoma gigantocellulare,

glandular carcinoma, granulosa cell carcinoma, hair-matrix carcinoma, haematoid carcinoma, hepatocellular carcinoma, Hurthle cell carcinoma, hyaline carcinoma, hypemephroid carcinoma, infantile embryonal carcinoma, carcinoma in situ, intraepidermal carcinoma, intraepithelial carcinoma, Krompecher's carcinoma, Kulchitzky-cell carcinoma, large-cell carcinoma, lenticular carcinoma, carcinoma lenticulare, lipomatous carcinoma, lymphoepithelial carcinoma, carcinoma medullare, medullary carcinoma, melanotic carcinoma, carcinoma molle, mucinous carcinoma, carcinoma muciparum, carcinoma mucocellulare, mucoepidermoid carcinoma, carcinoma mucosum, mucous carcinoma, carcinoma myxomatodes, naspharyngeal carcinoma, oat cell carcinoma, non-small cell carcinoma, carcinoma ossificans, osteoid carcinoma, papillary carcinoma, periportal carcinoma, preinvasive carcinoma, prickle cell carcinoma, pultaceous carcinoma, renal cell carcinoma of kidney, reserve cell carcinoma, carcinoma sarcomatodes, schneiderian carcinoma, scirrhous carcinoma, carcinoma scroti, signet-ring cell carcinoma, carcinoma simplex, small-cell carcinoma, solanoid carcinoma, spheroidal cell carcinoma, spindle cell carcinoma, carcinoma spongiosum, squamous carcinoma, squamous cell carcinoma, string carcinoma, carcinoma telangiectaticum, carcinoma telangiectodes, transitional cell carcinoma, carcinoma tuberosum, tuberous carcinoma, verrucous carcinoma, and carcinoma villosum.

[0076] The term "carcinoma"also encompasses adenocarcinomas. Adenocarcinomas are carcinomas that originate in cells that make organs which have glandular (secretory) properties or that originate in cells that line hollow viscera, such as the gastrointestinal tract or bronchial epithelia. Examples include, but are not limited to, adenocarcinomas of the breast, lung, pancreas and prostate.

[0077] Additional cancers encompassed by the present invention include, for example, Hodgkin"s Disease, Non-Hodgkin"s lymphoma, multiple myeloma, neuroblastoma, rhabdomyosarcoma, primary thrombocytosis, primary macroglobulinemia, small-cell lung tumors, primary brain tumors, malignant pancreatic insulanoma, malignant carcinoid, urinary bladder cancer, premalignant skin lesions, gliomas, testicular cancer, thyroid cancer, esophageal cancer, genitourinary tract cancer, malignant hypercalcemia; endometrial cancer, adrenal cortical cancer, mesothelioma and medulloblastoma.

[0078] In one embodiment of the present invention, the cancer is a hormone dependent prostate cancer. In another embodiment the cancer is a prostate cancer resistant to hormone blocking drugs. In yet another embodiment the effects of chemotherapeutic agents used for the treatment of prostate cancer is enhanced.

[0079] In still a further embodiment of the invention the cancer is selected from the group of : solid tumours, renal cell carcinoma, breast cancer, NSCLC, acute myeloid carcinoma, colorectal cancer, lung

carcinoma, melanoma, ovarian cancer, pancreatic carcinoma, cervix epitheloid carcinoma, breast adenocarcinoma, glioblastoma-astrocytoma, cervical carcinoma, human promyelocytic leukemia, or multidrug resistant versions thereof.

[0080] *PHARMACEUTICAL COMPOSITIONS*

[0081] The antisense oligonucleotide may be administered as a pharmaceutical composition with an appropriate pharmaceutically physiologically acceptable carrier, diluent, excipient or vehicle. The pharmaceutical compositions may also be formulated to contain the antisense oligonucleotide and one or more other chemotherapeutic agents for concurrent administration to a patient.

[0082] The pharmaceutical compositions of the present invention may be administered orally, topically, parenterally, by inhalation or spray or rectally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants and vehicles. The term parenteral as used herein includes subcutaneous injections, intravenous, intramuscular, intrasternal injection or infusion techniques.

[0083]

The pharmaceutical compositions may be in a form suitable for oral use, for example, as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsion hard or soft capsules, or syrups or elixirs. Compositions intended for oral use may be prepared according to methods known to the art for the

manufacture of pharmaceutical compositions and may contain one or more agents selected from the group of sweetening agents, flavouring agents, colouring agents and preserving agents in order to provide pharmaceutically elegant and palatable preparations. Tablets contain the active ingredient in admixture with suitable non-toxic pharmaceutically acceptable excipients including, for example, inert diluents, such as calcium carbonate, sodium carbonate, lactose, calcium phosphate or sodium phosphate; granulating and disintegrating agents, such as corn starch, or alginic acid; binding agents, such as starch, gelatine or acacia, and lubricating agents, such as magnesium stearate, stearic acid or talc. The tablets can be uncoated, or they may be coated by known techniques in order to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glyceryl monosterate or glyceryl distearate may be employed.

[0084] Pharmaceutical compositions for oral use may also be presented as hard gelatine capsules wherein the active ingredient is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate or kaolin, or as soft gelatine capsules wherein the active ingredient is mixed with water or an oil medium such as peanut oil, liquid paraffin or olive oil.

[0085] Aqueous suspensions contain the active compound in admixture with suitable excipients including, for example, suspending agents,

such as sodium carboxymethylcellulose, methyl cellulose, hydropropylmethylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents such as a naturally-occurring phosphatide, for example, lecithin, or condensation products of an alkylene oxide with fatty acids, for example, polyoxyethyene stearate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example, hepta-decaethyleneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol for example, polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example, polyethylene sorbitan monooleate. The aqueous suspensions may also contain one or more preservatives, for example ethyl, or *n*-propyl *p*-hydroxy-benzoate, one or more colouring agents, one or more flavouring agents or one or more sweetening agents, such as sucrose or saccharin.

[0086]

Oily suspensions may be formulated by suspending the active ingredients in a vegetable oil, for example, arachis oil, olive oil, sesame oil or coconut oil, or in a mineral oil such as liquid paraffin. The oily suspensions may contain a thickening agent, for example, beeswax, hard paraffin or cetyl alcohol. Sweetening agents such as those set forth above, and/or flavouring agents may be added to provide palatable oral preparations. These compositions can be

preserved by the addition of an anti-oxidant such as ascorbic acid.

[0087] Dispersible powders and granules suitable for preparation of an aqueous suspension by the addition of water provide the active compound in admixture with a dispersing or wetting agent, suspending agent and one or more preservatives. Suitable dispersing or wetting agents and suspending agents are exemplified by those already mentioned above. Additional excipients, for example sweetening, flavouring and colouring agents, may also be present.

[0088] Pharmaceutical compositions of the invention may also be in the form of oil-in-water emulsions. The oil phase may be a vegetable oil, for example, olive oil or arachis oil, or a mineral oil, for example, liquid paraffin, or it may be a mixtures of these oils. Suitable emulsifying agents may be naturally-occurring gums, for example, gum acacia or gum tragacanth; naturally-occurring phosphatides, for example, soy bean, lecithin; or esters or partial esters derived from fatty acids and hexitol, anhydrides, for example, sorbitan monoleate, and condensation products of the said partial esters with ethylene oxide, for example, polyoxyethylene sorbitan monoleate. The emulsions may also contain sweetening and flavouring agents.

[0089] Syrups and elixirs may be formulated with sweetening agents, for example, glycerol, propylene glycol, sorbitol or sucrose. Such formulations may also contain a demulcent, a preservative, and/or

flavouring and colouring agents.

[0090] The pharmaceutical compositions may be in the form of a sterile injectable aqueous or oleaginous suspension. This suspension may be formulated according to known art using suitable dispersing or wetting agents and suspending agents such as those mentioned above. The sterile injectable preparation may also be sterile injectable solution or suspension in a non-toxic parentally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Acceptable vehicles and solvents that may be employed include, but are not limited to, water, Ringer's solution, lactated Ringer's solution and isotonic sodium chloride solution. Other examples are, sterile, fixed oils which are conventionally employed as a solvent or suspending medium, and a variety of bland fixed oils including, for example, synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

[0091] Other pharmaceutical compositions and methods of preparing pharmaceutical compositions are known in the art and are described, for example, in "Remington: The Science and Practice of Pharmacy,"Gennaro, A., Lippincott, Williams & Wilkins, Philidelphia, PA (2000) (formerly "Remingtons Pharmaceutical Sciences").

[0092] *EFFICACY OF THE COMPOSITIONS*

[0093] 1. *In vitro* Testing

[0094] Initial determinations of the efficacy of the compositionsof the present invention may be made using *in vitro* techniques if required.

[0095] For example, the compositions can be tested *in vitro* by determining their ability to inhibit anchorage-independent growth of tumour cells. Anchorage-independent growth is known in the art to be a good indicator of tumourigenicity. In general, anchorage-independent growth is assessed by plating cells from an appropriate cancer cell-line onto soft agar and determining the number of colonies formed after an appropriate incubation period. Growth of cells treated with the compositionscan then be compared with that of cells treated with an appropriate control (as described above) and with that of untreated cells.

[0096] In one embodiment of the present invention, *in vitro* testing of the antisense oligonucleotide is conducted in a human cancer cell-line. Examples of suitable cancer cell-lines for *in vitro* testing of the compositions of the present invention include, but are not limited to, non-small cell lung carcinoma cell-lines A549 and H1299, breast cancer cell-line MCF-7, colon cancer cell-lines CaCo, HCT116 and HT29, cervical cancer cell-line HeLa, prostate cancer cell lines PC-3 and DU145. Other examples of suitable cell-lines are known in the art.

[0097]

If necessary, the toxicity of the compositionscan also be initially

assessed *in vitro* using standard techniques. For example,human primary fibroblasts can be treated *in vitro* with the oligonucleotide in the presence of a commercial lipid carrier such as lipofectamine. Cells are then tested at different time points following treatment for their viability using a standard viability assay, such as the trypan-blue exclusion assay. Cells are also assayed for their ability to synthesize DNA, for example, using a thymidine incorporation assay, and for changes in cell cycle dynamics, for example, using a standard cell sorting assay in conjunction with a fluorocytometer cell sorter (FACS).

[0098] 2. *In vivo* Testing

[0099] The ability of the compositions to inhibit tumour growth or proliferation *in vivo* can be determined in an appropriate animal modelusing standard techniques known in the art (see, for example, Enna, et al., Current Protocols in Pharmacology, J. Wiley & Sons, Inc., New York, NY).

[0100] In general,current animal models for screening anti-tumour compounds are xenograft models, in which a human tumour has been implanted into an animal. Examples of xenograft models of human cancer include, but are not limited to, human solid tumour xenografts in mice, implanted by sub-cutaneous injection and used in tumour growth assays; human solid tumour isografts in mice, implanted by fat pad injection and used in tumour growth assays; experimental models of lymphoma and leukaemia in mice, used in

survival assays, and experimental models of lung metastasis in mice.

[0101] For example, the compositions can be tested *in vivo* on solid tumours using mice that are subcutaneously grafted bilaterally with a pre-determined amount of a tumour fragment on day 0. The animals bearing tumours are mixed before being subjected to the various treatments and controls. In the case of treatment of advanced tumours, tumours are allowed to develop to the desired size, animals having insufficiently developed tumours being eliminated. The selected animals are distributed at random into groups that will undergo the treatments or act as controls. Suitable groupings would be, for example, those receiving the antisense alone, those receiving the chemotherapeutic agent(s) alone, those receiving antisense and chemotherapeutic and those receiving no treatment. Animals not bearing tumours may also be subjected to the same treatments as the tumour-bearing animals in order to be able to dissociate the toxic effect from the specific effect on the tumour. Chemotherapy generally begins from 3 to 22 days after grafting, depending on the type of tumour, and the animals are observed every day. The combinations of the present invention can be administered to the animals, for example, by bolus infusion. The different animal groups are weighed about 3 or 4 times a week until the maximum weight loss is attained, after which the groups are weighed at least once a week until the end of the trial.

[0102] The tumours are measured about 2 or 3 times a week until the tumour reaches a pre-determined size and / or weight, or until the animal dies if this occurs before the tumour reaches the pre-determined size / weight. The animals are then sacrificed and the tissue histology, size and / or proliferation of the tumour assessed.

[0103] For the study of the effect of the compositions on leukaemias, the animals are grafted with a particular number of cells, and the anti-tumour activity is determined by the increase in the survival time of the treated mice relative to the controls.

[0104] To study the effect of the compositions of the present invention on tumour metastasis, tumour cells are typically treated with the composition *ex vivo* and then injected into a suitable test animal. The spread of the tumour cells from the site of injection is then monitored over a suitable period of time by standard techniques.

[0105] *In vivo* toxic effects of the oligonucleotides can be evaluated by measuring their effect on animal body weight during treatment and by performing haematological profiles and liver enzyme analysis after the animal has been sacrificed.

[0106] *Table 1: Examples of xenograft models of human cancer*

[0107]

Cancer Model	*Cell Type*
Tumour Growth Assay. Human solid tumour xenografts in mice (sub-	Prostate (PC-3, DU145). Breast (MDA-MB-231, MVB-9). Colon (HT-29). Lung (NCI-H460, NCI-H209). Pancreatic (ASPC-1, SU86.86). Pancreatic: drug resistant (BxPC-

cutaneous injection)	3). Skin (A2058, C8161). Cervical (SIHA, HeLa-S3). Prostate Carcinoma. Cervical: drug resistant (HeLa S3-HU-resistance). Liver (HepG2). Brain (U87-MG). Renal (Caki-1, A498). Ovary (SK-OV-3).
Tumour Growth Assay. Human solid tumour isografts in mice (fat pad injection)	Breast: drug resistant (MDA-CDDP-S4, MDA-MB435-To.1)
Survival Assay. Experimental model of lymphoma and leukaemia in mice	Human: Burkitts lymphoma (Non-Hodgkin"s) (raji). Murine: erythroleukemia (CB7 Friend retrovirus-induced)
Experimental model of lung metastasis in mice	Human: melanoma (C8161). Murine: fibrosarcoma (R3)

[0108] *CLINICAL TRIALS IN CANCER PATIENTS*

[0109] One skilled in the art will appreciate that, following the demonstrated effectiveness of the combinations of the present invention *in vitro* and in animal models, they should be tested in Clinical Trials in order to further evaluate their efficacy in the treatment of cancer and to obtain regulatory approval for therapeutic use. As is known in the art, clinical trials progress through phases of testing, which are identified as Phases I, II, III, and IV.

[0110] Initially the compositions will be evaluated in preclinical studies in animal models (for pharmacology and toxicology) followed by a

Phase I trial. Typically Phase I trials are used to determine the best mode of administration (for example, by pill or by injection), the frequency of administration, and the toxicity for the compounds. Phase I studies frequently include laboratory tests, such as blood tests and biopsies, to evaluate the effects of a compound in the body of the patient. For a Phase I trial, a small group of cancer patients are treated with a specific dose of the antisense oligonucleotide and the one or more chemotherapeutic agent(s). During the trial, the dose is typically increased group by group in order to determine the maximum tolerated dose (MTD) and the dose-limiting toxicities (DLT) associated with the compound. This process determines an appropriate dose to use in a subsequent Phase II trial.

[0111] A Phase II trial can be conducted to evaluate further the effectiveness and safety of the compositions. In Phase II trials, the composition is administered to groups of patients with either one specific type of cancer or with related cancers, using the dosage found to be effective in Phase I trials.

[0112] Phase III trials focus on determining how a compound compares to the standard, or most widely accepted, treatment. In Phase III trials, patients are randomly assigned to one of two or more "arms". In a trial with two arms, for example, one arm will receive the standard treatment (control group) and the other arm will receive treatment with the composition of the present invention (investigational

group).

[0113] Phase IV trials are used to further evaluate the long-term safety and effectiveness of a compound. Phase IV trials are less common than Phase I, II and III trials and will take place after the composition has been approved for standard use.

[0114] *Eligibility of Patients for Clinical Trials*

[0115] Participant eligibility criteria can range from general (for example, age, sex, type of cancer) to specific (for example, type and number of prior treatments, tumor characteristics, blood cell counts, organ function). Eligibility criteria may also vary with trial phase. For example, in Phase I and II trials, the criteria often exclude patients who may be at risk from the investigational treatment because of abnormal organ function or other factors. In Phase II and III trials additional criteria are often included regarding disease type and stage, and number and type of prior treatments.

[0116]

Phase I cancer trials usually comprise 15 to 30 participants for whom other treatment options have not been effective. Phase II trials typically comprise up to 100 participants who have already received chemotherapy, surgery, or radiation treatment, but for whom the treatment has not been effective. Participation in Phase II trials is often restricted based on the previous treatment received. Phase III trials usually comprise hundreds to thousands of participants. This large number of participants is necessary in order

to determine whether there are true differences between the effectiveness of the composition of the present invention and the standard treatment. Phase III may comprise patients ranging from those newly diagnosed with cancer to those with extensive disease in order to cover the disease continuum.

[0117] One skilled in the art will appreciate that clinical trials should be designed to be as inclusive as possible without making the study population too diverse to determine whether the treatment might be as effective on a more narrowly defined population. The more diverse the population included in the trial, the more applicable the results could be to the general population, particularly in Phase III trials. Selection of appropriate participants in each phase of clinical trial is considered to be within the ordinary skills of a worker in the art.

[0118] *Assessment of patients prior to treatment*

[0119] Prior to commencement of the study, several measures known in the art can be used to first classify the patients. Patients can first be assessed, for example, using the Eastern Cooperative Oncology Group (ECOG) Performance Status (PS) scale. ECOG PS is a widely accepted standard for the assessment of the progression of a patient"s disease as measured by functional impairment in the patient, with ECOG PS 0 indicating no functional impairment, ECOG PS 1 and 2 indicating that the patients have progressively greater functional impairment but are still ambulatory and ECOG

PS 3 and 4 indicating progressive disablement and lack of mobility.

[0120] Patients" overall quality of life can be assessed, for example, using the McGill Quality of Life Questionnaire (MQOL) (Cohen et al (1995) Palliative Medicine *9*: 207-219). The MQOL measures physical symptoms; physical, psychological and existential well-being; support; and overall quality of life. To assess symptoms such as nausea, mood, appetite, insomnia, mobility and fatigue the Symptom Distress Scale (SDS) developed by McCorkle and Young ((1978) Cancer Nursing *1*: 373-378) can be used.

[0121] Patients can also be classified according to the type and/or stage of their disease and/or by tumor size.

[0122] *Administration of the compositions of the present invention in Clinical Trials*

[0123] The antisense oligonucleotide and the one or more chemotherapeutuc agent(s) are typically administered to the trial participants parenterally. In one embodiment, the composition is administered by intravenous infusion. Methods of administering drugs by intravenous infusion are known in the art. Usually intravenous infusion takes place over a certain time period, for example, over the course of 60 minutes.

[0124] *Monitoring of Patient Outcome*

[0125] The endpoint of a clinical trial is a measurable outcome that indicates the effectiveness of a treatment under evaluation. The

endpoint is established prior to the commencement of the trial and will vary depending on the type and phase of the clinical trial. Examples of endpoints include, for example, tumour response rate the proportion of trial participants whose tumour was reduced in size by a specific amount, usually described as a percentage; disease-free survival the amount of time a participant survives without cancer occurring or recurring, usually measured in months; overall survival the amount of time a participant lives, typically measured from the beginning of the clinical trial until the time of death. For advanced and/or metastatic cancers, disease stabilisation the proportion of trial participants whose disease has stabilised, for example, whose tumour(s) has ceased to grow and/or metastasise, can be used as an endpoint. Other endpoints include toxicity and quality of life.

[0126]

Tumour response rate is a typical endpoint in Phase II trials. However, even if a treatment reduces the size of a participant's tumour and lengthens the period of disease-free survival, it may not lengthen overall survival. In such a case, side effects and failure to extend overall survival might outweigh the benefit of longer disease-free survival. Alternatively, the participant's improved quality of life during the tumour-free interval might outweigh other factors. Thus, because tumour response rates are often temporary and may not translate into long-term survival benefits for the participant, response rate is a reasonable measure of a treatment's

effectiveness in a Phase II trial, whereas participant survival and quality of life are typically used as endpoints in a Phase III trial.

[0127] *PHARMACEUTICAL KITS*

[0128] The present invention additionally provides for therapeutic kits containing the antisense oligonucleotide and one or more chemotherapeutic agents in pharmaceutical compositions for use in the treatment of cancer. Individual components of the kit would be packaged in separate containers and, associated with such containers, can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

[0129] When the components of the kit are provided in one or more liquid solutions, the liquid solution can be an aqueous solution, for example a sterile aqueous solution. In this case the container means may itself be an inhalant, syringe, pipette, eye dropper, or other such like apparatus, from which the composition may be administered to a patient.

[0130] The components of the kit may also be provided in dried or lyophilised form and the kit can additionally contain a suitable solvent for reconstitution of the lyophilised components. Irrespective of the number or type of containers, the kits of the

invention also may comprise an instrument for assisting with the administration of the composition to a patient. Such an instrument may be an inhalant, syringe, pipette, forceps, measured spoon, eye dropper or any such medically approved delivery vehicle.

[0131] The disclosure of all patents, publications, including published patent applications, and database entries referenced in this specification are specifically incorporated by reference in their entirety to the same extent as if each such individual patent, publication, and database entry were specifically and individually indicated to be incorporated by reference.

[0132] To gain a better understanding of the invention described herein, the following examples are set forth. It should be understood that these examples are for illustrative purposes only. Therefore, they should not limit the scope of this invention in any way.

[0133] *EXAMPLES*

[0134] *Example 1: Pharmacokinetics and Metabolism in Animals*

[0135] The pharmacokinetics (PK) of SEQ ID NO:1 (and related oligonucleotide metabolites) were determined in rats and monkeys after single intravenous bolus injections of at escalating doses. In addition, the toxicokinetics and/or tissue distribution of SEQ ID NO:1 (and related metabolites) were determined as part of acute (24-hour) and repeat dose (14- and/or 21-days) continuous intravenous infusion toxicity studies in both rats and monkeys. The

plasma and tissue analyses were conducted by an appropriately validated (and cross-validated) capillary electrophoresis (CE) method.

[0136] *Absorption Pharmacokinetics in the rat*

[0137] Groups of Sprague-Dawley rats were administered single intravenous bolus injections of the SEQ ID NO:1 at doses of 10, 25 and 50 mg/kg (59, 147.5, and 295 mg/m^2). In each dose group, blood samples were collected from the animals (2 rats/sex/timepoint) at 5, 10, 20, 30, 45 min, and 2, 4, 8 and 24 h post dose The plasma was prepared for each sample for determination of SEQ ID NO:1 (and metabolites n+1 and n-1 to n-8) concentration.

[0138] SEQ ID NO:1 and metabolites were measurable in plasma of the animals in each dose group up to 24 h post dose. Based on AUC and C_{max} parameters, the plasma levels of SEQ ID NO:1 and its metabolites increased in proportion to administered dose. For SEQ ID NO:1, C_{max} values were achieved at the first sampling time (5 min) post dose while the maximum metabolite concentrations appeared as a plateau ranging from 5 to 10 min post dose. The elimination of SEQ ID NO:1 from plasma was biphasic with an initial rapid distribution phase followed by a more prolonged apparent terminal elimination phase ($t_{Â½}$, 4.72 to 5.80 h). The plasma clearance of SEQ ID NO:1 ranged from 49.67 to 43.30 mL/kgâ□¢h. The longer elimination $t_{Â½}$ with reduced plasma clearance tended

to occur at the higher dose, suggesting that the elimination pathways may be saturated at that dose level in the rat.

[0139] The pharmacokinetics of SEQ ID NO:1 (and metabolites) were also determined as part of a repeat dose toxicity study in rats. For this portion of the study, 8 rats/sex were assigned as satellite animals and administered SEQ ID NO:1 at a dose of 50 mg/kg/day (295 mg/m^2) (reduced to 40 mg/kg/day (236 mg/m^2) by continuous intravenous infusion for 14 days. The concentrations of SEQ ID NO:1 (and metabolites n+1 and n-1 through n-8) were measured in the plasma, with the results of the plasma analyses used for determination of pharmacokinetic parameters.

[0140] Based on the plasma SEQ ID NO:1 (and metabolites) concentrations during infusion, there were no apparent differences in the levels between males and females. Based on the mean plasma concentrations during infusion, steady state levels (C_{ss}) were achieved after approximately 24 h of continuous infusion: SEQ ID NO:1, 34.8 Âµg/mL; metabolites, 64.2 Âµg/mL. Elimination $t_{Â½}$, for SEQ ID NO:1 was calculated to be 8.6 h. The plasma clearance (Cl), calculated on the basis of C_{ss} and infusion rate was 47.8 mL/kgâ□¢h. The mean C_{max} values for SEQ ID NO:1 and SEQ ID NO:1 metabolites (n+1 and n-1 to n-8) were 40.4 and 82.2 Âµg/mL, respectively. The median time at which C_{max} occurred was 96 h for both SEQ ID NO:1 and SEQ ID NO:1 metabolites.

[0141] *Pharmacokinetics in the monkey*

[0142] Two groups of Cynomolgus monkeys were administered single intravenous injections of SEQ ID NO:1 as doses of 10 mg/kg (123 mg/m^2) and 50 mg/kg (615 mg/m^2). Serial blood samples were withdrawn from each animal at 0 (prior to dosing), 10, 20, 60,90 min and 2, 3, 6, 8 and 24 h post injection. Plasma concentrations of SEQ ID NO:1 (and metabolites, n+1 and n-1 to n-8) were determined, and the results of the plasma levels were used for determination of the pharmacokinetic parameters.

[0143] Based on non-compartmental analysis, the plasma elimination of SEQ ID NO:1 was determined to be biphasic in each treatment group. For each monkey, the C_{max} and AUC estimates were proportional with the administered dose for both SEQ ID NO:1 and its metabolites. T_{max} (observed) of SEQ ID NO:1 and metabolites was generally recorded at the first blood sampling timepoint (10 min post dosing) in all animals, except one male in the high dose group where the T_{max} (observed) for metabolites was recorded at the second blood sampling timepoint (20 min post dosing). There was a slightly increased mean Cl value and significantly larger mean elimination $t_{Â½}$ for the high dose group, compared to the low dose group. There were no clear observed sex differences in the pharmacokinetics of SEQ ID NO:1.

[0144] The toxicokinetics of SEQ ID NO:1 (and metabolites) were also determined as part of the repeat dose (14-day and 21-day) toxicity studies in monkeys. In the repeat dose study, groups of male and

female monkeys were administered SEQ ID NO:1 by continuous intravenous infusion for 14-days (Part 1) and 21-days (Part 2) at dose levels of 10, 20 and 40 mg/kg/day (123, 246, and 492 mg/m^2/day) (Part 1) and 2, 10 and 50 mg/kg/day (24.6, 123, and 615 mg/m^2/day) (Part 2). In Part 1, serial blood samples were withdrawn from each animal at 0 (pretreatment), 8, 48, 168 and 336 h following the onset of infusion, and from the recovery animal (male, high dose only) at 20, 60, 90 and 180 min post end of infusion. In Part 2, serial blood samples were withdrawn from each animal at 0 (pretreatment), 8, 24, 48, 96, 168, 336 and 480 h following the onset of infusion, and from the recovery animals (1/sex, high dose only) at 20, 60, 90 and 180 min post end of infusion.

[0145]

Based on the results in both Part 1 and 2, there were no apparent sex-differences in the toxicokinetic profile of SEQ ID NO:1 and its metabolites. In both Parts 1 and 2, the time to Css for the test article was consistently achieved at the first or second blood collection timepoint in all treatment groups. The estimates of elimination $t_{1/2}$ in the recovery monkeys were found to be consistent between the one Part 1 monkey, and the two Part 2 monkeys, ranging from 2.2 to 2.5 hours. However, considerable interindividual variability was found for the plasma Cl of SEQ ID NO:1, where values ranged from 45.7 to 116.7 mL/kgâ□¢h, with no apparent correlation to dose level or duration of infusion. AUC

estimates, for both SEQ ID NO:1 and its summed metabolites, were proportional with the duration of infusion and the administered dose level in the Part 1 and Part 2 Recovery animals.

[0146] *Tissue Distribution*

[0147] The tissue distribution of SEQ ID NO:1 (and metabolites) was determined in rats and monkeys as part of the repeat dose toxicity studies in those species. In general, following continuous infusion, the distribution of SEQ ID NO:1 (and metabolites) in both rats and monkeys was consistent with observations reported for other phosphorothioate oligonucleotides. The highest concentrations of SEQ ID NO:1 (and metabolites) were observed in the kidney > liver > spleen >lymph node (monkey) > lung (monkey) > heart. The levels in the brain were very low or below the limits of detection in both species suggesting that SEQ ID NO:1 (and metabolites) did not significantly cross the blood brain barrier.

[0148] The results of the repeat dose toxicity evaluations indicated that in both species the tissues manifesting the histopathological abnormalities included the kidney, liver, and lymph nodes. Since the highest concentrations of SEQ ID NO:1 and metabolites were found in those tissues, these data suggest that there was a relationship between the concentration of parent oligonucleotide (and metabolites) with morphological and with functional changes in those tissues. Upon discontinuation of SEQ ID NO:1 treatment, there was evidence in monkeys that both the parent compound and

its metabolite levels in various tissues decreased over time.

[0149] *Metabolism*

[0150] The principal metabolic pathway for oligonucleotides is cleavage via endo- and exonucleases (Cossum et al., 1993; Cossum et al., 1994; Iversen, 1991). Metabolism mediated by exo-and endonucleases results in shorter oligonucleotides and, ultimately, nucleosides that are degraded by normal metabolic pathways. The pattern of metabolites suggests primarily exonuclease activity with perhaps modest contributions by endonucleases.

[0151] *Excretion*

[0152] Phosphorothioate oligonucleotides are primarily eliminated in urine, with as much as 40% eliminated in 24 hours and up to 70% eliminated in 240 hours (Agrawal 1991; Zhang 1995; Iverson 1991; Srinivasan 1995; Grindel 1998). Fecal excretion is a minor pathway of elimination (Agrawal 1991; Zhang 1995). Oligonucleotides are excreted in urine mainly in a degraded form, although some intact oligonucleotide has been detected in urine at higher doses (â¥ 30mg/kg) (Agrawal 1991).

[0153] *EXAMPLE 2: Toxicology*

[0154] *Single Dose Toxicity*

[0155] *Acute intravenous toxicity study of SEQ ID NO:1 in Rats*

[0156] The purpose of this study was to assess the adverse effects of

SEQ ID NO:1 when administered as a single intravenous dose to Sprague-Dawley rats. In this study, four groups of animals (3/sex/group) were administered SEQ ID NO:1 by continuous intravenous infusion for 24-hours at escalating doses. Subsequent dose levels were incrementally escalated as follows when toxicological effects were not observed at the 40 mg/kg/day dose: 60, 80 and 90 mg/kg. Parameters assessed included mortality, clinical observations, body weight and food consumption assessment, clinical pathology and urinalysis measurements, and gross examination at necropsy.

[0157] The results indicated some test article related effects were found in animals that received doses of 60, 80 and 90 mg/kg.

[0158] *Acute intravenous toxicity study of SEQ ID NO:1 in the Monkey*

[0159] The objective of this single, dose escalating study was to establish a maximum tolerated dose (MTD) for SEQ ID NO:1 and to assess the effect of administered SEQ ID NO:1 on the cardiac function of conscious Cynomolgus monkeys. In this study, three monkeys (two males and one female) were administered SEQ ID NO:1 by continuous intravenous infusion for 24-hours at escalating doses of 10, 20, 40 and 80 mg/kg. There was a 3-day washout between doses. One female animal, administered vehicle only (PBS) in the same manner, was used as a control. Electrocardiogram (ECG) measurements were conducted on each animal (including control) prior to, during and after the end of each infusion interval. Clinical

signs, mortality, body weight and food consumption measurements, hematology, coagulation and clinical chemistry parameter evaluations, were recorded and evaluated. In addition, blood samples were removed prior to and at the end of each infusion interval to measure complement (CH50 and Bb). Blood samples were also drawn at the end of each infusion for analysis of parent, SEQ ID NO:1.

[0160] There were no deaths, clinical signs, body weight changes, or effects on food consumption. There were no treatment-related effects on ECG recordings and blood pressure. There was an apparent increasing trend in activated partial thromboplastin times (APTT) following the last dose (Day 14) in all animals; however, all values were within normal ranges. The complement analyses indicated that up to 40 mg/kg, the Bb and CH50 values were within the reference range. At the high dose (80 mg/kg), the complement values from one animal (female) were outside the normal range (higher Bb and lower CH50) suggesting that complement activation had occurred. Analysis of plasma samples obtained at the end of infusion indicated that the concentrations of SEQ ID NO:1 increased with escalating dose levels. These effects indicate an apparent inhibition of the intrinsic coagulation pathway and modest actuation of the alternative complement pathway response. These treatment-related changes were typical class effects of phosphorothioate oligonucleotide administration.

[0161] *Repeat Dose Toxicity*

[0162] *A 14-day continuous intravenous infusions toxicity study of SEQ ID NO:1 in the rat with a 14-day recovery*

[0163] The objective of this study was to assess the potential adverse effects of SEQ ID NO:1 in male and female Sprague Dawley rats when administered by continuous intravenous infusion for 14 days. Ten rats/sex/group were administered SEQ ID NO:1 at doses of 0 (control), 2, 10, or 50 mg/kg/day. Animals in the control group received the vehicle article, PBS. However; due to severe adverse clinical signs and mortality of animals in the 50 mg/kg/day dose group, the high dose was reduced to 40 mg/kg/day on days 8, 9, and 10. An additional 5 rats/group were included in the control and high dose group as recovery animals, and were allowed a 14-day observation period following the treatment period. Parameters assessed during the study include mortality, clinical signs, body weights, food consumption, ophthalmoscopic examination, clinical pathology assessment (hematology, coagulation, clinical chemistry, and urinalysis). Terminal procedures included a complete necropsy of each animal, and histopathologic evaluation of selected tissues for animals in the control and high dose groups. For toxicokinetic evaluations, an additional 8 rats/sex were included in the high dose groups. Serial and terminal blood samples were withdrawn from satellite animals at selected time points during infusion and after the end of infusion. Designated tissues were also collected from

selected toxicokinetic animals and analyzed for SEQ ID NO:1 (and metabolites) concentration.

[0164]

Treatment-related effects were found in the high dose group, including high morbidity and mortality, reduced body weights and reduced food consumption. Clinical pathology results showed dose-dependent anemia, thrombocytopenia, coagulopathic selectivity for APTT, and liver and kidney toxicity in both sexes. Pathological findings strongly correlated with these results, and showed major treatment-related changes in numerous tissues and organs of the high dose animals, in most tissues and organs of the mid dose animals; and sporadically in the evaluated tissues and organs of the low dose animals. The adverse effects to SEQ ID NO:1 treatment appeared more pronounced in males than females suggestive of an apparent sex effect. The toxicokinetic results from animals infused at the high dose level indicated that SEQ ID NO:1 C_{ss}, achieved at approximately 24 h after start of infusion, was 34.8 Âµg/mL. The apparent $t_{Â½}$ was 8.6 h and the total plasma clearance was 46.9 mL/kgâ□¢h. Tissue uptake of SEQ ID NO:1 (and metabolites) was highest in the kidney followed by the liver; spleen and heart. The levels in the brain were undetectable. This pattern of tissue distribution was considered typical of phosphorothioate oligonucleotides. Many of the adverse effects that were found in the animals of this study appeared to correlate to the high levels of SEQ ID NO:1 (and metabolites) in the kidney, liver

and spleen.

[0165] *Repeat dose (14- and 21-day) toxicity study in Monkeys with a 14 or 21-day recovery period*

[0166] The objective of this study was to assess the toxicity and toxicokinetics of SEQ ID NO:1 in male and female Cynomolgus monkeys after continuous intravenous infusion for 14 (Part 1) or 21 (Part 2) days. The reversibility of potential toxic effects of SEQ ID NO:1 at the highest dose level was also assessed during a 14 or 21-day recovery period. In the 14-day study (Part 1), groups of one male and one female monkey recovered daily doses of 0 (vehicle, PBS), 10, 20 or 40 mg/kg of SEQ ID NO:1 by continuous intravenous infusion for 14 consecutive days. An additional male was assigned to each of the vehicle and high dose group and remained on study for 14-day recovery period. In the 21-day study (Part 2), four groups of three male and three female monkeys received daily doses of 0 (vehicle, PBS), 2, 10, or 50 mg/kg of SEQ ID NO:1 by continuous intravenous infusion for 21 consecutive days. An additional male and female were assigned to each of the control and high dose groups, and remained on study for 21 days after conclusion of dosing. Parameters evaluated for Part 1 and 2 included clinical signs; body weights, food consumption, appetite, clinical pathology assessment (hematology, clinical chemistry, coagulation, and urinalysis), ECG assessment, opthalmoscopy examinations, and immunology measurements (complement split

products Bb analysis). Blood and tissue samples (at necropsy) were collected and analyzed for SEQ ID NO:1 (and metabolites) concentration. At termination, surviving animals were euthanized and subjected to macroscopic and microscopic examination.

[0167] In Part 1 (14-day infusion up to 40 mg/kg/day), there were no deaths, no treatment-related clinical signs, effects on appetite, ophthalmology effects, cardiology, hematology effects, or changes in organ weights. There was a slight decrease in body weight in one mid and high male, however, it was unclear if it was related to SEQ ID NO:1 administration. A reversible increase in activated partial thromboplastin time (APTT) was found in the high dose animals on Day 14. Bb appeared to increase in the high dose group on Day 14. Transient changes in some clinical parameters were noted in mid and low dose animals but were not considered toxicologically significant. Treatment-related macroscopic and histopathological changes were noted in the liver, kidneys, lymph nodes, infusion sites, and adrenals (high dose only). In the recovery animal, similar but less severe effects were noted in the kidneys, lymph nodes and infusion site.

[0168] In Part 2, one high dose animal was sacrificed on Day 19 for ethical reasons. Treatment-related reversible clinical signs were limited to a few high dose animals, mainly males, and were reflective of weakness (e.g., decreased activity, decrease appetite, pallor, cold to touch). A slight decrease in body weight was observed in some

high dose males; however, at the end of the recovery period both high dose animals had gained weights. There was a significant decrease in appetite noted in the majority of high dose males, in addition to a slight decrease in one high dose female. Bilateral retinopathy was noted in one high dose male at the end of the treatment period, but due to the low incidence, the significance of this finding is unclear. A marked increase in WBC counts was observed on Day 20 in all high dose animals, which was associated with high neutrophil, monocyte, and/or large unstained cell counts. In addition, slight to moderate reductions in red blood cell, hematocrit, and hemoglobin values, suggestive of anemia were noted in three high dose animals, along with one animal in the mid and low dose groups in addition to two control animals. APTT values were significantly longer on Day 20 in animals from the high dose group, compared to control values. Changes in organ weights were limited to a slight increase in relative and absolute kidney weights, which were noted in two high dose females along with the recovery male. Treatment-related histopathology changes were noted in the liver, kidneys, adrenals (high dose only), lymph nodes (mid and high-dose), brain (high dose only), heart (high-dose only), thymus, and infusion sites. In the kidneys, the severity of findings was dose-related. In the recovery animals, similar but less severe changes were noted in the kidneys, liver, and lymph nodes. In addition minimal to moderate lymphoid atrophy was noted in all groups, including the control and recovery animals. Because of its

increased incidence and severity in the high dose group, this finding was considered to be an indirect effect of SEQ ID NO:1.

[0169] In summary, the administration of SEQ ID NO:1 for 14 days at 10, 20 or 40 mg/kg/day produced partially reversible treatment-related effects, that were limited to prolongation of activated partial thromboplastin time (40 mg/kg/day), and microscopic changes in various tissues and organs (all groups). Administration of SEQ ID NO:1 for 21 days at 50 mg/kg/day was associated with reversible signs of weakness, decreases in body weight and appetite, prolongation of APTT, anemia, thrombocytopenia, and monocytosis. This dose level also resulted in increased kidney weight, along with microscopic changes in various organs that were partially reversible after a 21-day recovery period. Treatment-related changes at 2 and 10 mg/kg/day were limited to slight anemia and multiorgan microscopic changes. Most of the treatment related effects noted were similar and consistent with those observed in monkey studies for compounds of the same chemical class.

[0170] *In vitro Hemolysis*

[0171] SEQ ID NO:1 injection was tested for its potential to cause hemolytic activity based on cell lysis and hemoglobin release in human whole blood. Four milliliters of each concentration of dosing solution (1.0, 5.0, and 10 mg/mL), 0.99% saline (negative control), or 1% saponin (positive control) were mixed with 5.0 mL of diluted

blood and incubated for one hour at 37 Â± 1Â°C under static and dynamic conditions. Following the test procedures, the hemolytic index was calculated. The test article was non-hemolytic under both static and dynamic conditions. None of the test article concentrations had a hemolytic index of greater than 2.

[0172] *EXAMPLE 3: Effects of SEQ ID NO:1 on PC-3 Prostate Tumor Growth in SCID Mice*

[0173] PC-3 human prostatic cancer cells ($1X10^7$ cells in 100 Âµl of PBS) were subcutaneously injected into the right flank of 6-7 week old male SCID mice. After the tumor size reached an approximate volume of 50 mm^3, 14 days post tumor cell injection, SEQ ID NO:1 was administered by bolus infusion into the tail vein every other day at 10 mg/kg. Control animals received saline alone for the same period. Treatments lasted for 36 days thereafter. Antitumor activities were estimated by the inhibition of tumor volume, which was measured with a caliper on six different occasions over 36-day period. Each point represents mean tumor volume calculated from 5 animals per experimental group. As illustrated in Figure 1A, SEQ ID NO:1 treatment demonstrated strong inhibitory effects on the growth of human prostate carcinoma.

[0174] DU145 human prostatic cancer cells ($1X10^7$ cells in 100 Âµl of PBS) were subcutaneously injected into the right flank of 6-7 week old male SCID mice. After the size of the tumors reached an approximate volume of 50 mm^3, 13 days post tumor cell injection,

SEQ ID NO:1 was administered by bolus infusion into the tail vein every other day at 10 mg/kg. Control animals received saline alone for the same period. Treatments lasted for 30 days thereafter. Antitumor activities were estimated by the inhibition of tumor volume, which was measured with a caliper on nine different occasions over 30-day period. Each point represents mean tumor volume calculated from 5 animals per experimental group. As illustrated in Figure 1B, SEQ ID NO:1 treatment demonstrated strong inhibitory effects on the growth of human prostate carcinoma.

[0175] *EXAMPLE 4: Effects of Combination Therapy on Prostate Tumor Growth in SCID Mice*

[0176] Figure 2 shows results from two independent experiments. In both experiments, DU145 human prostatic cancer cells ($1X10^7$ cells in 100 Î¼l of PBS) were subcutaneously injected into the right flank of 6-7 weeks old male SCID mice. After the size of tumor reached an approximate volume of 50 mm^3, 13 (upper panel) or 11 (lower panel) days post tumor cell injection, SEQ ID NO:1 was administered by bolus infusion into the tail vein every other day at 10 mg/kg 15 times (upper panel) or 14 times (lower panel), respectively. Control animals received saline alone for the same period. Antitumor effect of SEQ ID NO:1 was further compared to that of mitoxantrone (novantrone Â®) alone or in combination. Mitoxantrone was administered intravenously once at the beginning

of the treatments at a dose of 2 mg/kg (upper panel) or once a week for four weeks at a reduced dose of 0.8 mg/kg (lower panel). All treatments were stopped at day 42 (upper panel) or 38 (lower panel), respectively. A day after the last treatment, tumors were excised from the animals and their weights were measured. A standard bar graph (Figure 2) was used to demonstrate the differences in tumor weights with each bar representing mean tumor weight calculated from 5 (upper panel) or 10 (lower panel) animals. As illustrated in the left panel, SEQ ID NO:1 treatments resulted in significant delay of tumor growth compared to saline control. The delay in tumor growth achieved with SEQ ID NO:1 was superior to the inhibitory effects observed with mitoxantrone alone. The combination of SEQ ID NO:1 with mitoxantrone (SEQ ID NO:1 +) showed some additive antitumor effects. In the lower panel, mitoxantrone alone resulted in delay of tumor growth and the combination therapy was significantly more potent than mitoxantrone monotherapy.

[0177] *EXAMPLE 5: Immune Related Issues*

[0178] An issue that must inevitably be addressed when developing antisense therapeutics is whether the compound produces non-specific immune stimulation that is not a result of target sequence interactions. Immune stimulation can be the result of two properties of AS-ODN, one sequence specific and one backbone specific. Un-methylated CpG di-nucleotides, usually present in bacterial DNA,

stimulate innate immune responses in vertebrates and can further augment acquired immune responses to both pathogens and tumor cells. The presence of un-methylated CpGs in an oligonucleotide can have the same effect if in an optimal sequence context. In addition, the phosphorothioate backbone, used in first generation antisense compounds, has been found to be immune stimulatory in a sequence independent manner. As shown in a number of tumor xenograft experiments, SEQ ID NO:1 is highly effective in SCID mice that are T and B cell deficient suggesting SEQ ID NO:1 acts independent of the acquired immune system. There is strong evidence from other studies that NK cells are stimulated by CpG motifs.

[0179] *Effects of SEQ ID NO:1 on Caki Renal Tumor Growth in SCID/beige Mice.*

[0180] To address whether SEQ ID NO:1 anti-tumor activity is NK mediated, tumor xenograft growth was assessed in SCID/beige mice that are NK, T and B cell deficient. Caki-1 human kidney cancer cells ($5X10^6$ cells in 100 Âµl of PBS) were subcutaneously injected into the right flank of 6-7 weeks old female SCID/beige mice. After the size of tumor reached an approximate volume of 100 mm^3, 7 days post tumor cell injection, SEQ ID NO:1 and SEQ ID NO:1-SCR were administered (10 mg/kg/2days, i.v.). Control animals received saline alone for the same period. Caliper measurements at 1week intervals were used to calculate tumor

volumes. Each point in Figure 3 (top) represents mean tumor volume calculated from 10 animals per experimental group. After 32 days the mice were sacrificed and the tumors weighed. Each bar in Figure 3 (bottom) represents the mean tumor weight and standard error calculated for each treatment group. SEQ ID NO:1 was highly effective against renal tumor xenografts in these mice. Other studies have demonstrated that the anti-tumor efficacy of immuno-stimulatory CpG ODNs is compromised in murine tumor models using these mice, consistent with SEQ ID NO:1 not acting via immune stimulation.

[0181] *EXAMPLE 6: Inhibition of R1 mRNA in Tumors*

[0182] Effects of SEQ ID NO:1 administration on R1 mRNA levels in HT-29 colon tumors in nude mice were investigated.

[0183] *Methods.*

[0184] For determination of mRNA levels in tumors by Northern Blot, total RNA was prepared from excised tumors using TRIzol reagent (GIBCO BRL). Northern blot analysis was performed as previously described (Hurta and Wright, 1995). RNA was subjected to electrophoresis through 1 % formaldehyde agarose gels followed by transfer to nylon membranes. Blots were hybridized in the presence of a R1 fragment (McClarty et al, 1987). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were simultaneously probed for RNA loading controls.

[0185] *Results.*

[0186] As shown in Figure 4, marked reduction in the R1 mRNA levels was observed in two independent HT-29 tumors at day 16 following administration of SEQ ID NO:1 every other day at a dose of 10 mg/kg. The results provide strong evidence that SEQ ID NO:1 is reaching the tumor site *in vivo* and is acting by an antisense mechanism of action.

[0187] SEQ ID NO:1 decreased R1 mRNA levels in HT-29 colon tumors xenografted into mice (Figure 4). Tumors of sufficient size were not obtainable for many tumor types and use of surrogate mouse tissue was not appropriate due to target sequence differences.

[0188] *EXAMPLE 7: Expression of R1 in Normal and Tumor Cell Lines*

[0189]

Methods. To measure R1 protein levels, western blot analysis was conducted. Briefly, cells were washed once with PBS and whole cell protein extracts were prepared in 50-150 Î¼l of 2 x sample loading buffer (100 mM Tris, pH 6.8, 200 mM DTT, 4% SDS, 20% glycerol and 0.015% bromphenol blue). Extracted protein (10-20 Î¼g) was fractionated on 12 % SDS-PAGE, transferred to nitrocellulose membranes and total protein visualized by India ink staining. R1 protein was detected with AD 203, an anti-R1-antibody (5-50 Î¼g/ml; obtained from either InRo Biomedtek, Sweden or Accurate Chemical and Scientific Corporation, Westbury, NY, USA) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG

(Sigma, St. Louis, MO) at a dilution of 1:5,000. The 80kDa R1 protein was visualized by development of the peroxidase reaction (ECL chemiluminescence, Amersham Corporation). GAPDH protein was detected as an internal control. WI-38 and HUVEC cells are normal cell lines. The remainder are tumor cell lines routinely used in xenograft tumor model studies.

[0190] *Results.*

[0191] Earlier studies have demonstrated elevated RNR levels and activity in tumors and tumor cell lines. To assess whether this is a general phenomenon of cancer cells, the R1 protein levels were examined in untreated cancer cell lines derived from diverse cancer types, including renal, skin, colon and breast cancer cell lines (Figure 5). The R1 expression was compared to R1 expression in 2 normal cell lines, WI38 and HUVEC. GAPDH protein expression was determined as an internal reference. Consistent with its role in cancer progression, R1 levels were elevated in all of the tumor cell lines tested. The increase in R1 varied from 1.4-14 fold, compared to HUVEC cells, and 1.8-17 fold, compared to WI-38 cells. These data support the targeting of R1 for down-regulation via antisense compounds.

[0192] R1 protein is over-expressed in a number of tumor cell lines making R1 a good tumor target (Figure 5).

[0193] *EXAMPLE 8: Inhibition of the Growth of Tumor Cell Lines*

[0194] The effect of SEQ ID NO:1 on the colony forming ability were evaluated in the following human tumor cell lines:

[0195] Hep G2 (liver) SK-OV-3 (ovary)

[0196] U-87 MG (brain) A2058 (melanoma)

[0197] H460 (lung) MDA-MB-231 (breast)

[0198] AsPC-1 (pancreas).

[0199] *Methods.* Tumor cells were washed in 5 ml of phosphate buffered saline, pH 7.2, prior to 0.2 Î¼M antisense oligonucleotide/lipofectin treatment for 4 hours. The medium was removed and the cells were gently washed with 5 ml of growth medium. The cells were then cultured in growth medium for seven to ten days. Surviving colonies were visualized by methylene blue staining and colonies of 50 or more cells were scored (Choy et al., 1988 and Huang and Wright, 1994). Results are summarized from 4 to 8 trials for each tumor cell line.

[0200] *Results.* A greater than 60% inhibition in colony forming ability was observed for all cell lines treated with SEQ ID NO:1 with the exception of the U-87 MG (brain), MDA-MB-231 (breast) and AsPC-1 (pancreas). A decrease of roughly 40 to 60% in colony forming ability was observed in these three cell lines following administration of antisense oligonucleotide.

[0201] SEQ ID NO:1 inhibited the growth of human tumor cell growth in

colony forming assays (Figure 6).

[0202] *EXAMPLE 9: Inhibition of the R1 Target at the mRNA level*

[0203] Northern blot analyses were performed to determine if SEQ ID NO:1 treatment of human tumor cell lines had an effect on R1 mRNA levels. Results of these assays demonstrate that SEQ ID NO:1 specifically decreases R1 mRNA .

[0204] *Methods.* Total RNA was isolated using TRIzol reagent (GIBCO BRL) after cells were treated with 0.2Î¼M SEQ ID NO:1 for four hours in the presence of cationic lipid (Lipofectin reagent, GIBCO BRL), washed with PBS, and incubated for 16 hours to recover in normal medium containing 10% FBS. Northern blot analysis was performed as previously described (Hurta and Wright, 1995). RNA was subjected to electrophoresis through 1 % formaldehyde agarose gels followed by transfer to nylon membranes. Blots were hybridized in the presence of a R1 fragment (McClarty et al, 1987). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were simultaneously probed for RNA loading controls.

[0205]

Results. A significant decrease in R1 mRNA was observed following SEQ ID NO:1 treatment of HT-29 (human colon adenocarcinoma) and MDA-MB-231 (human breast adenocarcinoma) cell lines. The results are shown below. Two independent treatments with SEQ ID NO:1 (1 and 2) consistently resulted in marked reduction in the R1 mRNA levels in both cell

lines.

[0206] Incubation of 0.2Î¼M SEQ ID NO:1 with human colon or breast adenocarcinoma cells decreased R1 mRNA levels in those cells (Figure 7).

[0207] *EXAMPLE 10: Inhibition of the R1 Target at the Protein Level*

[0208] *Immunoprecipitation*

[0209] Immunoprecipitation analyses were performed to determine if SEQ ID NO:1 treatment of human tumor cell lines had an effect on R1 protein expression. Results of these assays demonstrate that SEQ ID NO:1 specifically decreases R1 protein expression.

[0210] Methods. Immunoprecipitation was performed using a saturating amount of AD203 anti-R1 monoclonal antibody as previously described (Choy et al., 1988). Human tumor cells, AsPC-1 (pancreatic adenocarcinoma), were exposed for 4 hours to SEQ ID NO:1, SEQ ID NO:1 Mis (a SEQ ID NO:1 sequence containing four base mismatch) or SEQ ID NO:1 Scr (a sequence with the same ratio of ACTG as the SEQ ID NO:1 sequence but scrambled). Cells were then washed and labeled with ^{35}S-methionine for 4-7 hours. R1 protein was specifically immunoprecipitated with R1 antibody from cell lysate, resolved on sodium dodecyl sulfate-polyacrylamide gels and analyzed by autoradiography.

[0211] Results. Figure 8 shows the results. Newly synthesized R1 protein was specifically precipitated with R1 antibody in the cells that were

not treated with antisense oligonucleotides (Control). R1 protein expression, however, was dramatically decreased following exposure of tumor cells to 0.2 ÂµM SEQ ID NO:1 (SEQ ID NO:1). There was no significant decrease in R1 protein synthesis following administration of 0.2 ÂµM of either a SEQ ID NO:1 Scr or SEQ ID NO:1 Mis.

[0212] Immunoprecipitation analysis using R1 specific antibody to measure the synthesis of R1 protein in the AsPC-1 pancreatic adenocarcinoma cells demonstrated that 0.2ÂµM SEQ ID NO:1 specifically inhibit the expression of R1 protein. In contrast, incubation with a SEQ ID NO:1 sequence with a four base mismatch or an oligonucleotide with the same ratio of ACTG as SEQ ID NO:1 but scrambled did not decrease R1 protein expression (Figure 8).

[0213] *Western Blots*

[0214] Western blots were performed to determine if SEQ ID NO:1 treatment of human tumor cell lines had an effect on R1 protein expression. Results of these assays demonstrate that SEQ ID NO:1 specifically decreases R1 protein expression in a dose-dependent manner.

[0215]

Methods. MDA-MB-231 human breast adenocarcinoma cells were treated with increasing concentrations (0.025-0.2 ÂµM) of SEQ ID NO:1, 0.2 ÂµM of a scrambled control analogue of SEQ ID NO:1

(SEQ ID NO:1 Scr) or a mismatched control analogue of SEQ ID NO:1 (SEQ ID NO:1 Mis) that contains four base changes. Cells were then washed and fresh media were added. Cells were harvested 8-18 hours later for protein extractions. Aliquots of cell extracts were heated at 100°C for 5 minutes and then analyzed on sodium dodecyl sulfate-polyacrylamide gels (Choy et al., 1988). Proteins were then transferred to membranes. Membranes were blocked and then incubated with anti-R1 antibody for 1 hour at room temperature. Membranes were washed three times in cold TBS-Tween buffer followed by incubation for 30 minutes to 1 hour at room temperature in the presence of a second antibody (goat anti-rabbit immunoglobulin linked with horseradish peroxidase). Blots were washed and bound antibodies were detected by development of the alkaline phosphatase reaction (Fan et al., 1996).

[0216] *Results.* The results in Figure 9 show that R1 protein expression decreased in a dose-dependent manner following exposure of tumor cells to increasing concentrations of SEQ ID NO:1. There was no decrease in R1 protein following the administration of 0.2Î¼M of either a scrambled version of SEQ ID NO:1 (SEQ ID NO:1 Scr) or a four base pair mismatch of SEQ ID NO:1 (SEQ ID NO:1 Mis). Densitometric measurements of each band are expressed as a relative intensity as illustrated below.

[0217] Incubation of increasing concentrations of SEQ ID NO:1 (0.025 to

0.2ÂµM) with human breast adenocarcinoma cells decreased R1 protein expression in a dose-dependent manner. In contrast, incubation with a SEQ ID NO:1 sequence with a four base mismatch or an oligonucleotide with the same ratio of ACTG as SEQ ID NO:1 but scrambled did not decrease R1 protein expression (Figure 9).

[0218] *EXAMPLE 11: Target-Specific Inhibition of R1 mRNA Expression by SEQ ID NO:1*

[0219] In order to examine the specificity of inhibition of R1 mRNA by SEQ ID NO:1, northern blot analyses of other cellular RNA levels in A2058 human melanoma cells treated with SEQ ID NO:1 or a scrambled control analogue of SEQ ID NO:1 were carried out.

[0220] *Methods.* A2058 human melanoma cells, grown to subconfluency (70-80%), were treated with 0.2ÂµM of phosphorothioate antisense ODNs for 4 hr in the presence of cationic lipid (Lipofectin reagent, final concentration, 5Î¼g/ml, GIBCO BRL) and Opti-MEM (GIBCO BRL). Cells were washed once with PBS and incubated for 16 hr in Î±-MEM medium (GIBCO BRL) containing 10% FBS. Total RNA was prepared in TRIzol reagent (GIBCO BRL) and northern blot analysis was performed as previously described (Hurta and Wright, J. Cell. Biochem. 57: 543-56, 1995) with some modifications. RNA prepared from cells treated with lipofectin alone (Control), SEQ ID NO:1 and scrambled control analogue (SEQ ID NO:1 Scr) were subjected to electrophoresis through 1% formaldehyde agarose

gels followed by transfer to nylon membrane. The blots were hybridized with ^{32}P-labeled probes that detect R1 mRNA, 28S rRNA, 18S rRNA, thioredoxin mRNA, Âµ-actin mRNA, GAPDH mRNA, thioredoxin reductase mRNA, ribosomal protein S9 mRNA, RNase MRP RNA, RNase P RNA and R2 mRNA.

[0221] *Results.* Because no sequence similarities exist between SEQ ID NO:1 target sequence and any of the RNA sequences we selected, SEQ ID NO:1 was not expected to affect the expression of these unrelated cellular RNAs, if SEQ ID NO:1 indeed inhibit R1 mRNA expression target-specifically. As shown in Figure 10, SEQ ID NO:1 treated cells showed a significant decrease in R1 mRNA but not other RNAs. Furthermore, SEQ ID NO:1 reduced R1 mRNA levels in a highly sequence-specific manner, since no effects were observed on expression of R1 and other cellular RNAs in cells treated with SEQ ID NO:1 scramble control sequence.

[0222] SEQ ID NO:1 was found to significantly decrease expression of R1 mRNA in a highly target-specific and sequence-specific manner. No effects were observed on expression of other cellular RNAs including 28S rRNA, 18S rRNA, thioredoxin mRNA, Ã□-actin mRNA, GAPDH mRNA, thioredoxin reductase mRNA, ribosomal protein S9 mRNA, RNase MRP RNA, RNase P RNA and R2 mRNA, in cells treated with SEQ ID NO:1 or its scramble control sequence (Figure 10).

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Claims

[c1] 1. A use of an antisense oligonucleotide comprising SEQ ID NO:1 alone or in combination with a chemotherapeutic for the treatment of prostate cancer.

Antisense oligonucleotides directed to ribonucleotide reductase R1 and uses thereof in the treatment of cancer

Abstract

The present invention provides combinations of antisense oligonucleotides directed to a mammalian ribonucleotide reductase R1 gene alone or in combination with one or more chemotherapeutic agents for use in the treatment of cancer. The compositions of the present invention are effective in decreasing the growth and/or metastasis of cancer cells, including drug resistant cancer cells.

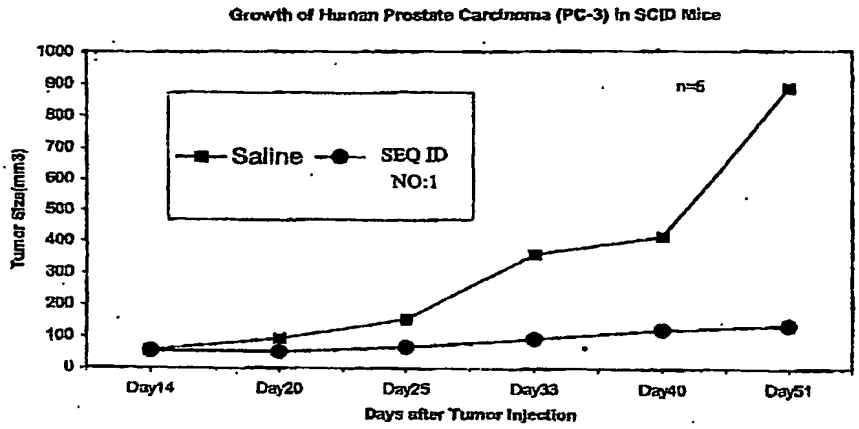

Growth of Human Prostate Carcinoma (PC-3) in SCID Mice
Tumor Size(mm3)
1000
900
800
700
600
500
400
300
200
100
0
n=6
Saline
SEQ ID NO:1
Day14
Day20
Day25
Day33
Day40
Day51
Days after Tumor Injection

Figure 1A

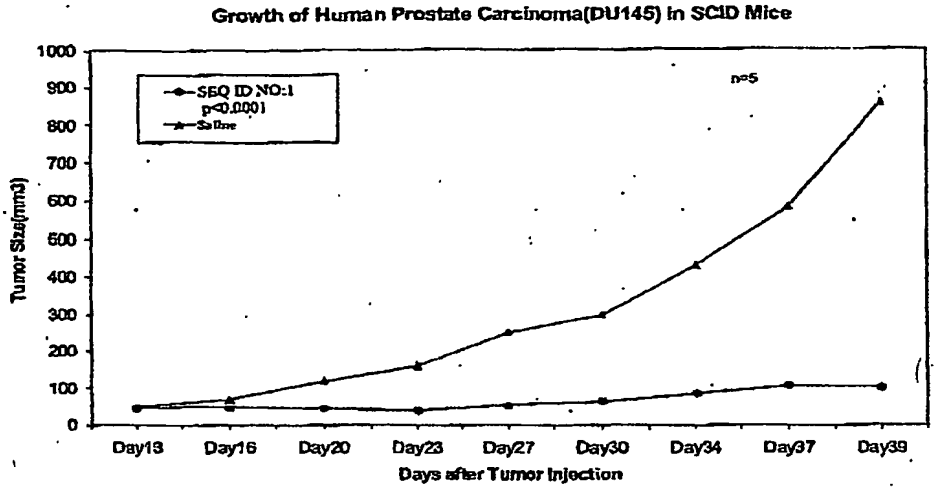

Growth of Human Prostate Carcinoma(DU145) in SCID Mice
n=5
SEQ ID NO:1
p<0.0001
Saline
Tumor Size(mm3)
1000
900
800
700
600
500
400
300
200
100
0
Day13
Day16
Day20
Day23
Day27
Day30
Day34
Day37
Day39
Days after Tumor Injection

Figure 1B

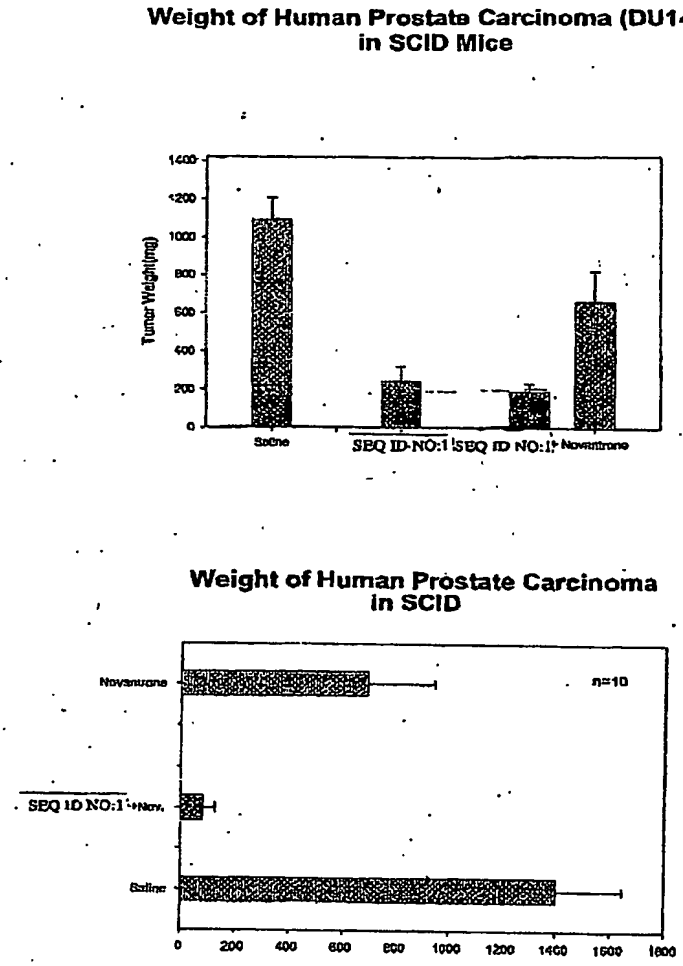
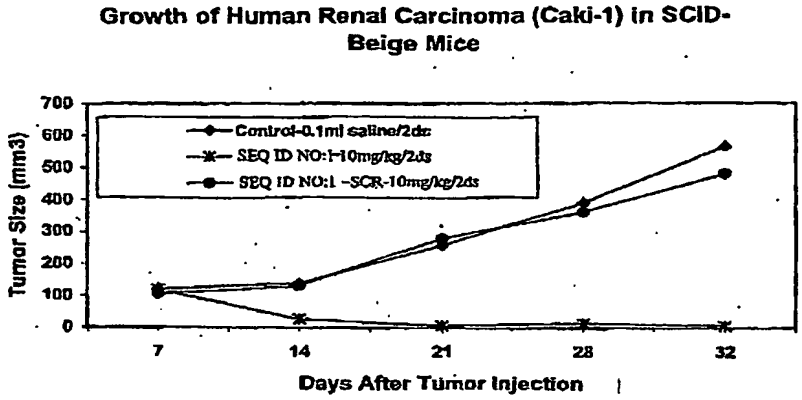

Weight of Human Prostate Carcinoma (DU145)
in SCID Mice
Tumor Weight(mg)
1400
1200
1000
800
600
400
200
0
Saline
SEQ ID NO:1
SEQ ID NO:1+ Novantrone
Weight of Human Prostate Carcinoma
in SCID
Novantrone
n=10
SEQ ID NO:1+Nov.
Saline
0
200
400
600
800
1000
1200
1400
1600
1800

Figure 2


Growth of Human Renal Carcinoma (Caki-1) in SCID-Beige Mice
Control-0.1ml saline/2ds
SEQ ID NO:1-10mg/kg/2ds
SEQ ID NO:1 -SCR-10mg/kg/2ds
Tumor Size (mm3)
700
600
500
400
300
200
100
0
7
14
21
28
32
Days After Tumor Injection

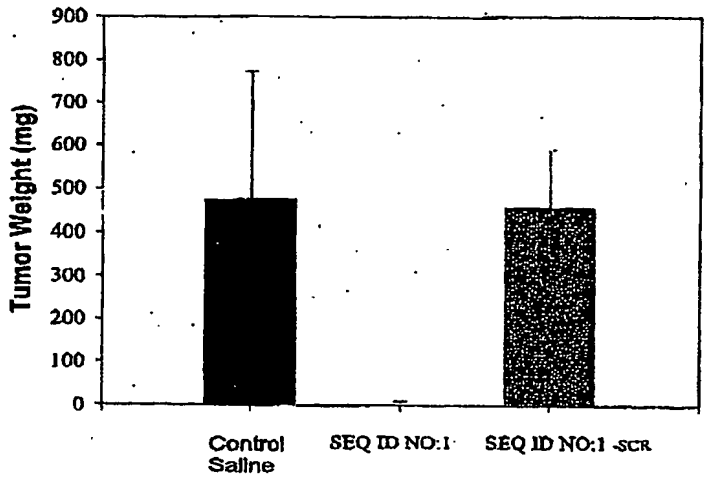

Weight of Human Renal Carcinoma (Caki-1) in SCID-Beige Mice
Tumor Weight (mg)
900
800
700
600
500
400
300
200
100
0
Control Saline
SEQ ID NO:1
SEQ ID NO:1 -SCR

Figure 3

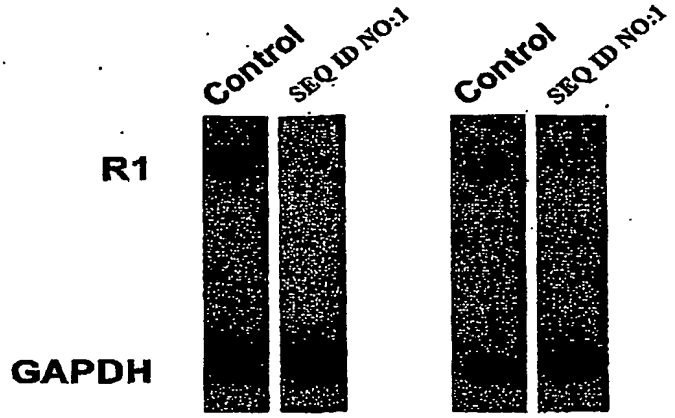


Figure 4

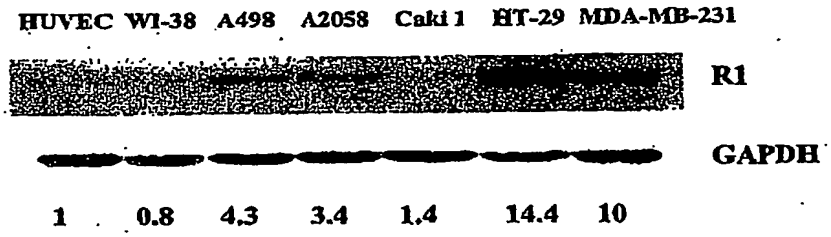


Figure 5
Over-expression of R1 in Tumor Cell Lines

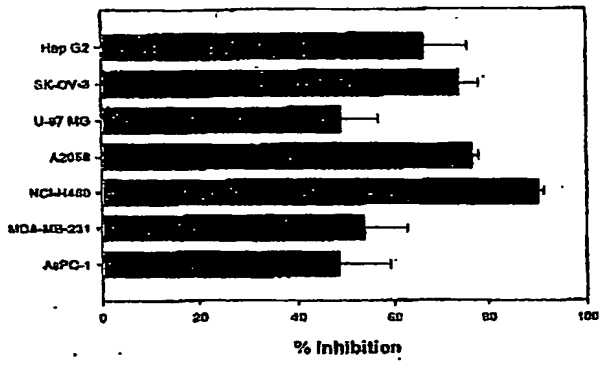
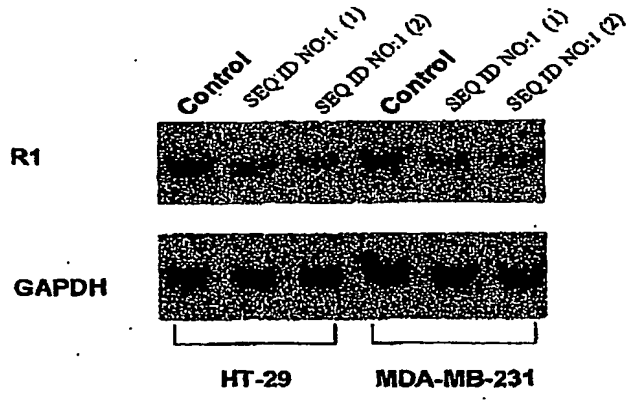


Figure 6
Inhibition of Colony Forming Ability with 0.2 μM SEQ ID NO:1



Reduction in R1 mRNA Expression by SEQ ID NO:1

Figure 7

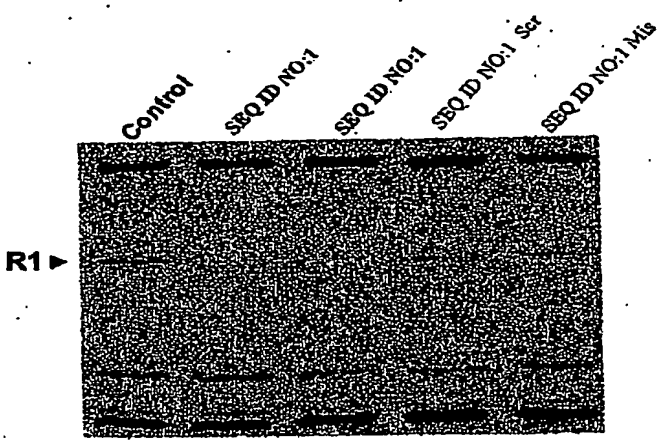
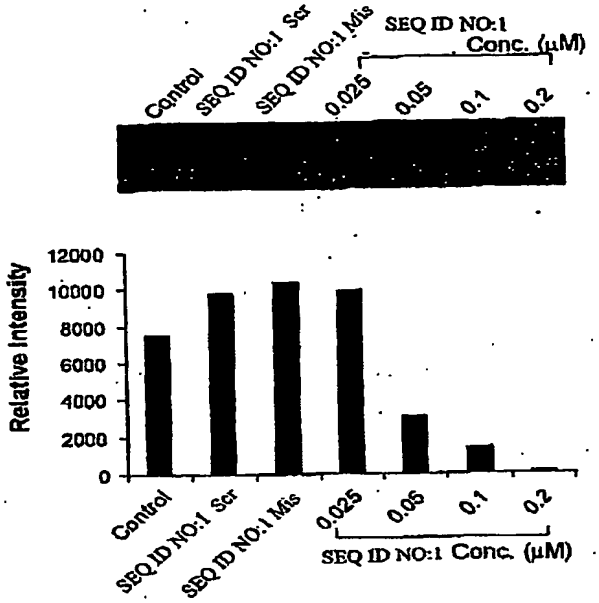


Figure 8
Reduction in R1 Protein Expression by SEQ ID NO:1



Dose-Dependent and Sequence-Specific Inhibition of R1 Protein Expression by SEQ ID NO:1

Figure 9.

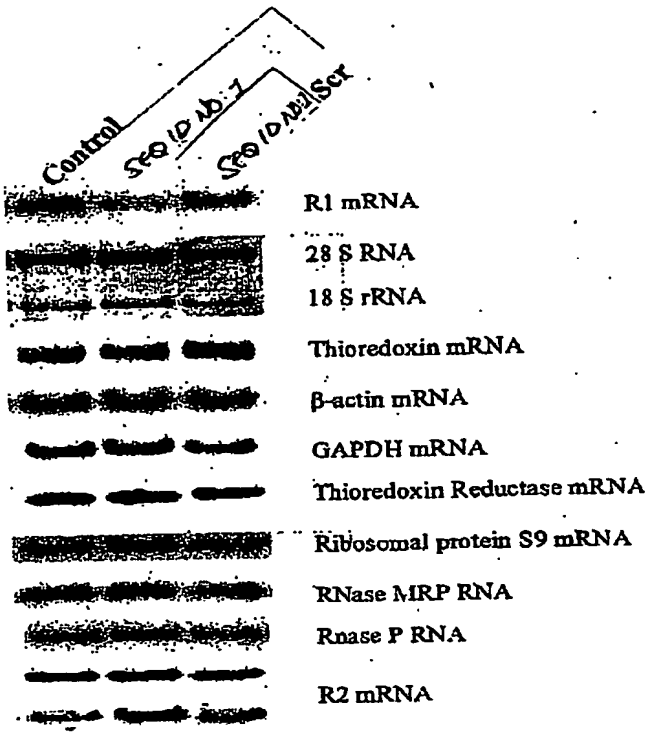


Figure 10
Target-Specific Inhibition of R1 mRNA